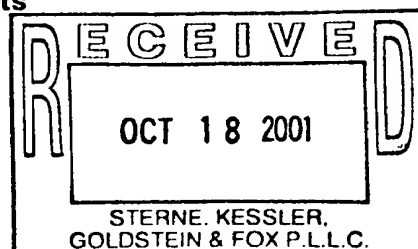




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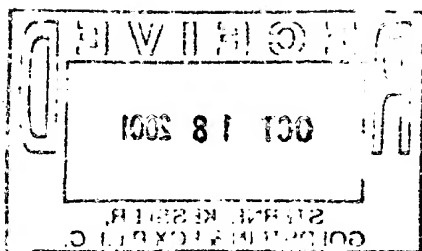
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55216 Ingelheim
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BOEHRINGER INGELHEIM INTERNATIONAL GMBH

55216 Ingelheim am Rhein (BRD)

Cyk-4 polypeptides, DNA molecules encoding them and
their use in screening methods

The present invention relates to a new protein Cyk-4, which is involved in cytokinesis, and to therapies interfering with cell division, in particular tumor therapy.

- 5 The process of cytokinesis produces two daughter cells from a single parental cell and permanently segregates the products of the cell division cycle. Cytokinesis is one of the few processes in biology known to require coordination between microtubules and actin filaments.
- 10 Indeed, in animal cells there are two steps in cytokinesis that rely on interactions between microtubules and the actin-based contractile ring (for review see (Field et al., 1999; Glotzer, 1997). In the first instance, the microtubule-based mitotic spindle
- 15 specifies the position of the contractile ring. This allows the division plane to be positioned so that the separated chromosomes are partitioned equally into the two daughter cells. Subsequently, after ingression of the cleavage furrow, there is a second step that
- 20 depends on both microtubules and the contractile ring. Completion of cytokinesis requires the central spindle, which contains bundled, antiparallel microtubules. The molecular mechanisms underlying these two microtubule dependent steps in cytokinesis are not known.
- 25 The degree to which the progression of cytokinesis depends on the central spindle varies somewhat in different experimental organisms. In invertebrate embryos, a transient interaction between astral microtubules of the mitotic spindle and the cell cortex
- 30 is sufficient to position the cleavage furrow

(Rappaport, 1985). Furrows specified in this manner ingress, but if the spindle is removed, these furrows do not usually complete cytokinesis (Rappaport, 1985). In contrast, in *Drosophila* spermatocytes, contractile
5 ring formation requires the central spindle (Giansanti et al., 1998). Similarly, in cultured mammalian cells, astral microtubules appear to be insufficient to induce furrow ingression, instead the presence or absence of a central spindle determines whether or not a cleavage
10 furrow forms (Cao and Wang, 1996; Eckley et al., 1997; Rieder et al., 1997; Savoian et al., 1999; Wheatley and Wang, 1996). Moreover, in cultured cells, the central spindle is also required for completion of cytokinesis (Wheatley and Wang, 1996). In *C. elegans* embryos, as in
15 invertebrate embryos, only the later stages of cytokinesis appear to depend on the central spindle. Embryos depleted of the kinesin-like protein ZEN-4/CeMKlp1 fail to assemble the central spindle, yet cleavage furrows form and ingress, but cytokinesis does
20 not proceed to completion (Powers et al., 1998; Raich et al., 1998). In summary, the initiation of cytokinesis depends on the central spindle in some but not in all organisms, whereas there appears to be a general requirement for the central spindle for the
25 completion of cytokinesis in animal cells. While it is clear that the central spindle plays an important role in cytokinesis the underlying mechanism remains elusive.

Cleavage furrow ingression is driven by the actin-based
30 contractile ring. Like many actin-based structures, the contractile ring requires the RhoA GTPase for its assembly. Rho family GTPases are thought to act as

molecular switches that cycle between inactive GDP-bound forms and active GTP-bound forms; their ability to exchange and hydrolyze GTP is regulated by additional factors, the so-called guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Inactivation of RhoA by the exoenzyme C3 (Aktories and Hall, 1989), inhibits cytokinesis in a wide variety of experimental settings by causing disassembly of cortical actin structures and the contractile ring (Drechsel et al., 1997; Kishi et al., 1993; Mabuchi et al., 1993; Moorman et al., 1996; O'Connell et al., 1999). Further, a Rho GEF is essential for cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999). GTP-bound RhoA interacts with a number of putative effectors including formins, Rho Kinase, Citron kinase, and a regulatory subunit of myosin phosphatase (for review see (Van Aelst and D'Souza-Schorey, 1997). The requirement for RhoA in cytokinesis may reflect its ability to regulate formins since members of the formin gene family are required for cytokinesis in budding yeast (BNI1/BNR1) (Imamura et al., 1997), fission yeast (Cdc12) (Chang et al., 1997), *Drosophila* (*dia*) (Castrillon and Wasserman, 1994) and *C. elegans* (*cyk-1*) (Swan et al., 1998). Several formins also bind to profilin (Chang et al., 1997 ; Evangelista et al., 1997; Imamura et al., 1997; Watanabe et al., 1997), a key regulator of actin polymerization. It is conceivable that GTP-bound RhoA promotes contractile ring assembly by activating actin polymerization via the formins and by activating myosin motor activity.

There is compelling evidence that the microtubule-based central spindle and the actin-based cleavage furrow are both essential for cytokinesis. Next, the question was asked how these two cytoskeletal polymers interact.

5 There are a few cases in which an interaction between the microtubule and actin cytoskeletal systems have been characterized. Examples include nuclear positioning in budding yeast (Carminati and Stearns, 1997; Fujiwara et al., 1999; Miller et al., 1999),
10 spindle orientation in epithelial cells (Busson et al., 1998) and in certain asymmetrically dividing cells, such as the posterior blastomere of the two cell *C. elegans* embryo (Gönczy et al., 1999a; Hyman and White, 1987; Skop and White, 1998; Waddle et al., 1994). In
15 these examples there is evidence that the dynein/dynactin microtubule motor complex may mediate the interaction of microtubules with the cell cortex.

It was an object of the invention to elucidate the microtubule dependent steps in cytokinesis. In
20 particular, it was sought to define the role of the central spindle in this process, in order to be able to interfere with this process and thus with cytokinesis, which provides a new approach for therapy, in particular cancer therapy.

25 Therefore, to solve the problem underlying the invention, it was investigated how the central spindle assembles and how it functions in cytokinesis.

In the course of the experiments, a novel gene, designated *cyk-4*, gene has been identified by genetic
30 analysis in *C. elegans*. Embryos from *cyk-4(t1689ts)*

mutant hermaphrodites were shown to initiate, but to fail to complete, cytokinesis. These embryos also fail to assemble the central spindle. It was shown that the *cyk-4* gene encodes a GTPase activating protein (GAP) for Rho family of GTPases. The Cyk-4 GAP domain activates GTP hydrolysis by RhoA, Rac1 and Cdc42 *in vitro*. RNA-mediated interference of RhoA, Rac1 and Cdc42 suggests that Rho plays an essential role in cytokinesis and is the target of Cyk-4 GAP activity for cytokinesis. It could be shown that Cyk-4 and a Cyk-4:GFP fusion protein localize to the central spindle and persist at cell division remnants. It was found that Cyk-4 localization is dependent on the kinesin-like protein Zen-4/CeMKlp1 and vice-versa. The data obtained in the present invention suggest that Cyk-4 and Zen-4/CeMKlp1 cooperate in central spindle assembly. Central spindle localization of Cyk-4 could accelerate GTP hydrolysis by RhoA thereby allowing contractile ring disassembly and completion of cytokinesis.

In further experiments, the biochemical state of CYK-4 and ZEN-4 has been examined and it was found that they do indeed associate *in vivo*. These proteins also efficiently associate *in vitro* and an *in vitro* binding assay has been used to molecularly dissect the regions of both proteins that are necessary and sufficient for this interaction. It is shown that the protein encoded by the *ts* allele *cyk-4(t1689)* is defective in binding to ZEN-4. The identification of mutations in *zen-4* that partially rescue *cyk-4(t1689)* and map to the CYK-4 binding region provides strong evidence for the importance of the interaction between CYK-4 and ZEN-4

for progression of cytokinesis. It has also been shown that the human orthologs of CYK-4 and ZEN-4, HsCYK-4/MgcRacGAP and MKLP1, are in a complex which has been purified. The complex contains approximately
5 stoichiometric amounts of HsCYK-4 and MKLP1. The human complex localizes to the central spindle and to division remnants, as do the nematode proteins. Thus, in the present invention, an evolutionarily conserved complex that is essential for central spindle assembly
10 has been identified.

In the present invention, the role of the *cyk-4* gene in the early divisions of the *C. elegans* embryo was characterized. It was shown that Cyk-4 is required for the late stages of cytokinesis. Interestingly, *cyk-4*
15 mutant embryos fail to assemble the central spindle. Positional cloning and localization studies revealed that the *cyk-4* gene encodes a novel GTPase activating protein (GAP) for the Rho family of GTPases that localizes to the central spindle. The missense
20 mutation in the *cyk-4(t1689ts)* allele is found in a domain dispensable for GAP activity suggesting that Cyk-4 may have another function in addition to activating GTP hydrolysis by Rho family proteins. Accordingly, it was found that Cyk-4 and the kinesin-
25 like protein Zen-4/CeMKlp1 are interdependent for their proper localization. Based on these data, a model is proposed by which Cyk-4 acts in concert with Zen-4/CeMKlp1 to assemble the central spindle. The concentration of Cyk-4 to the central spindle would
30 then serve to target the GAP domain to the fully ingressed contractile ring where it could promote GTP

hydrolysis by RhoA, thereby facilitating the completion of cytokinesis.

The division of a cell into two daughters requires dynamic interactions between the microtubule-based
5 mitotic spindle and the actin-based contractile ring. In animal cells the position of the cleavage furrow, an actomyosin-based structure, is determined by the mitotic spindle in a manner that is poorly understood. In recent years it has become clear that the central
10 spindle, an array of microtubule bundles that forms during anaphase, also plays an important role in cytokinesis. To gain insight into this fundamental cellular process, the cytokinesis-defective mutant, *cyk-4*, was analyzed. Cytological analysis reveals that
15 *cyk-4* mutant embryos fail to assemble the central spindle. Though lacking a central spindle, *cyk-4* mutant embryos furrow extensively, but they fail to complete cytokinesis. The mutation responsible for the *cyk-4* phenotype was found to be a missense mutation in
20 a gene encoding a Rho family GAP that, *in vitro*, stimulates GTP hydrolysis by Rho, Rac, and Cdc42. CYK-4 localizes to the central spindle and to cell division remnants. CYK-4 colocalizes with the ZEN-4/CeMKLP1 kinesin-like protein. Moreover, CYK-4 and ZEN-4/CeMKLP1
25 are interdependent for their localization. It is concluded that the CYK-4 GAP and the ZEN-4/CeMKLP1 kinesin-like protein cooperate to assemble the central spindle. Furthermore, it is proposed that a concentrated source of CYK-4 GAP on the central spindle
30 could downregulate the RhoA GTPase and thereby promote the late stages of cytokinesis.

The findings of the present invention provide a model for CYK-4 dependent assembly of the central spindle:

In *cyk-4* mutant embryos the robust microtubule bundles
5 that constitute the central spindle do not form.
Instead, the spindle develops into two mitotic asters
separated by a few overlapping, disorganized,
microtubules. A similar phenotype is observed in *zen-4*
mutant embryos (Powers et al., 1998; Raich et al.,
10 1998). Thus both the ZEN-4/CeMKLP1 kinesin-like protein
and the CYK-4 GAP are essential for this microtubule
bundling. The *Drosophila* orthologue of ZEN-4/CeMKLP1 is
also required for cytokinesis, though it seems to be
required for all aspects of furrow ingression (Adams et
15 al., 1998). Members of the MKLP1 subfamily of kinesin-
like proteins have microtubule bundling activity *in*
vitro (Kuriyama et al., 1994; Nislow et al., 1992).
However, *in vivo*, ZEN-4 mediated microtubule bundling
requires CYK-4.

20 In answering the question how CYK-4 and ZEN-4 could
cooperate to assemble the central spindle, it is
proposed that a complex containing multiple motor
proteins could specifically localize to overlapping,
antiparallel microtubules (figure 10). If such a motor
25 complex transits along a microtubule it might continue
to an end and dissociate. However, if such a motor
complex transited along a microtubule in the vicinity
of an antiparallel microtubule, the complex might bind
simultaneously to both microtubules and attempt to move
30 alternately in opposite directions, the net result

being that the complex would concentrate in the region of microtubule overlap. Since CYK-4 does not have a microtubule motor domain, yet it is essential for the formation of the central spindle, it is proposed that

5 CYK-4 forms a complex with multiple ZEN-4 homodimers that localizes to and stabilizes overlapping antiparallel microtubules (figure 10).

It was of interest to find out if the RhoGAP activity of CYK-4 necessary to promote microtubule bundling by

10 ZEN-4. It is proposed that central spindle assembly is unlikely to require CYK-4 GAP activity. This is suggested by two lines of evidence. First, central spindle assembly is defective in the *cyk-4(t1689ts)* allele that carries a missense mutation at amino

15 acid 15. This substitution is distant from the C-terminal GAP domain, and *in vitro*, the amino terminus of CYK-4 is dispensable for GAP activity. Thus this allele would be predicted to retain catalytic activity

20 *in vivo* and therefore GAP activity is not sufficient for central spindle assembly. Moreover, Rho RNAi experiments reveal that central spindle assembly is Rho independent, suggesting that Rho GAP activity is not required for this process. Thus CYK-4 may act to promote central spindle assembly, independent of its

25 GAP activity.

In the present invention, the function of the CYK-4 gap domain was analysed:

If CYK-4 function in central spindle assembly is independent of the Rho GTPase, it was of interest to

30 determine the function of the CYK-4 GAP domain. CYK-4

is likely bifunctional, one function being to promote the assembly of the central spindle, the second function being to promote GTP hydrolysis by Rho family members. These two functions might be related in that
5 the first function would serve to concentrate CYK-4 at a site where GAP activity is required. It may be assumed that CYK-4 GAP activity is required late in cytokinesis, to promote GTP hydrolysis by a Rho family GTPase whose downregulation causes disassembly of the
10 contractile ring and cell separation (fig. 10).

It was determined which GTPase might CYK-4 act on to promote cytokinesis. The CYK-4 GAP domain has all the hallmarks of a Rho family GAP and it may therefore be expected that it will act on this subfamily of the
15 GTPase superfamily. Like many other RhoGAPs, the GAP domain of CYK-4 is promiscuous in its ability to promote GTP hydrolysis on Rho, Rac, and Cdc42 (Lamarche and Hall, 1994). The strongest piece of evidence that CYK-4 acts on Rho is based on the observation that of
20 the GTPases tested, Rho is the only one that is clearly essential for cytokinesis. The requirement for Rho in cell division is well documented in a variety of experimental systems. To date there is no evidence that Rac is required for cytokinesis and our data using RNAi
25 to deplete Rac also failed to detect a role for this GTPase in this process. Moreover, it has been recently reported that *ced-10* mutants, which are defective in corpse engulfment subsequent to apoptosis and distal tip cell migration, contain mutations in the *rac* gene
30 (Reddien and Horvitz, 2000). *ced-10* mutants do not have any gross phenotypes indicative of a role in cytokinesis. Moreover, *Rac1* deficient mice are

gastrulation defective, but the embryos do not contain multinucleate cells indicative of a cell division defect (Sugihara et al., 1998). The sum of these data argue that Rac is not an essential target of CYK-4 during cytokinesis. With regard to Cdc42, previous studies have implicated this GTPase in cytokinesis (Drechsel et al., 1997; Dutartre et al., 1996). Superficially, the weakly penetrant cytokinesis phenotype observed in *Cdc42(RNAi)* embryos is consistent with these earlier data. However the *Cdc42(RNAi)* embryos that are cytokinesis defective are also osmotically swollen and therefore cytokinesis defect may be indirect. Thus at this juncture it appears most likely that RhoA is the key substrate for the CYK-4 GAP activity.

Further support for the hypothesis that completion of cytokinesis requires downregulation of RhoA by CYK-4 would be supported by experiments in which the *cyk-4* phenotype is phenocopied by RhoA mutants that are hydrolysis defective. However the genetic tools necessary to express such dominant mutants in the early *C. elegans* embryo are currently unavailable. It is surprising that the CYK-4 GAP domain is less active towards RhoA as compared to Rac or Cdc42, if indeed RhoA is the relevant target of its GAP activity. One possible explanation is that full length CYK-4 has a different activity profile as compared to the isolated GAP domain. A more interesting possibility is that CYK-4 localization is important for CYK-4 GAP activity. The phenotype of *cyk-4* mutant embryos suggests that CYK-4 needs to act when the contractile ring is in close proximity to the central spindle. Since CYK-4 is

concentrated on the central spindle at this time, its high local concentration might overcome its lower activity towards RhoA.

In the present invention, the central spindle was shown
5 to be at the center of cytokinesis:

There appear to be at least two microtubule-dependent steps in cytokinesis, contractile ring positioning and completion of cytokinesis. In some cells both processes are dependent on the central spindle. An
10 important open question is whether these two reactions are mechanistically similar. While assembly of the contractile ring requires activation of RhoA, it was previously shown that the position of the contractile ring is specified in a RhoA-independent manner in
15 *Xenopus* embryos (Drechsel et al., 1997). It has been shown here that a Rho GAP is required for the late stages of cytokinesis, suggesting that the second process, completion of cytokinesis, does involve RhoA. It is therefore assumed that the two microtubule-
20 dependent steps in cytokinesis are distinct.

CYK-4 and ZEN-4 are not the only components of the central spindle, a number of other components, some of which are required for cytokinesis, are also present at this site. Polo kinase is known to associate with MKLP1
25 and to concentrate in the central spindle (Adams et al., 1998; Lee et al., 1995), and this kinase is essential for cytokinesis. Rho associated kinase also localizes to this site (Kosako et al., 1999). The AIR-2 aurora-like kinase localizes to the central spindle
30 (Schumacher et al., 1998). This kinase seems to be

required primarily for chromosome segregation (Woollard and Hodgkin, 1999), its direct involvement in cytokinesis requires further analysis. INCENP and the TD-60 antigen also localize to the central spindle and
5 there is evidence that they may play a role in cytokinesis (Eckley et al., 1997; Mackay et al., 1998; Martineau-Thuillier et al., 1998; Savoian et al., 1999). Interestingly, a Rho GEF that is required for cytokinesis, ECT2, also accumulates on the central
10 spindle (Tatsumoto et al., 1999), however the *Drosophila* ortholog, Pebble, does not localize in this manner (Prokopenko et al., 1999).

Further analysis of the specific functions of all of these cytokinesis regulators is the basis to determine
15 which of these proteins are functionally interdependent as has been shown is the case for Cyk-4 and Zen-4/CeMKlp1.

Furthermore, it was shown that the central spindle is a structure that is essential for completion of
20 cytokinesis. Two proteins required for central spindle assembly, CYK-4 and ZEN-4, were shown to co-localize to the central spindle and to somehow act in concert with one another. In experiments of the present invention, it was demonstrated that in vivo, CYK-4 and ZEN-4 are
25 present in an evolutionarily conserved protein complex and the nature of this complex in *C. elegans* embryos and in human cells was characterized in detail. These data suggest that the CYK-4/ZEN-4 complex, which was named centralspindlin, consists of a two molecules each
30 of CYK-4 and ZEN-4. CYK-4 binds to the neck region of the ZEN-4 kinesin, raising the possibility that the

motor activity of the ZEN-4 is directly regulated by the CYK-4 RhoGAP.

Analytical biochemistry of the native centralspindlin complex isolated from *C. elegans* embryos demonstrates the existence of a complex containing the ZEN-4 kinesin and the CYK-4 RhoGAP. In vitro binding experiments were used to define the critical determinants for this interaction and to demonstrate that the two subunits are able to self associate. Genetic and biochemical suppression of the CYK-4 S15L mutation by a second site mutation in ZEN-4 strongly argues that the interaction between CYK-4 and ZEN-4 is critical for CYK-4 function. Indeed, in vivo, the majority of ZEN-4 is in a complex with CYK-4. Moreover, three lines of evidence indicate that HsCYK-4 and MKLP1 are in a tetrameric complex. First, immunopurification of HsCYK-4 and MKLP1 reveals equal amounts of the two proteins. Second, these two proteins co-migrate on sucrose density gradients with a similar S value as observed for the *C. elegans* proteins. Third, the two proteins precisely co-migrate on a gel filtration column and their fractionation behavior suggests a native molecular mass for the complex of ~300 kDa. Previous determinations of the native molecular mass of MKLP1 have been reported and the values are similar to those obtained in the present invention here (Chui et al., 2000; Kuriyama et al., 1994). Previous studies had not taken the presence of CYK-4 into consideration and therefore they interpreted their data to indicate that MKLP1 exists as a homotetramer. However, the data obtained in the experiments of the invention are not compatible with this interpretation, but they rather indicate that the

centralspindlin complex is a tetramer containing two molecules of the kinesin ZEN-4/MKLP1 and two molecules of the RhoGAP CYK-4.

CYK-4 binds to ZEN-4 in a particularly interesting
5 region of this kinesin family member. Vale and
colleagues Rice et al., 1999 have established that a
critical element of the kinesin molecule lies just
C-terminal to the catalytic core; the neck linker
region. ATP binding to one catalytic core induces a
10 large scale conformational change in the neck linker
region that causes the other catalytic core to extend
towards the adjacent tubulin dimer situated on the plus
side of the initial microtubule contact (Rice et al.,
1999). In the present invention, it was found that
15 CYK-4 binds to a region of ZEN-4 that includes the neck
linker region. In conventional kinesin, the neck linker
region corresponds to a region 15 amino acids long that
connects the catalytic core of kinesin to the coiled
coil stalk domain. Among the family of KIN-N motors,
20 the MKLP1 subfamily has a distinctly divergent neck
linker region, it lacks several nearly invariant
residues and the linker between the catalytic core and
the coiled coil region is about 5 times longer than in
other members on the KIN-N family. The divergence of
25 this critical region of the kinesin suggests that
MKLP1-mediated microtubule motility may differ from
that of other kinesins. Moreover, since CYK-4 binds to
the neck linker region of ZEN-4 it is possible that
CYK-4 binding may in fact regulate ZEN-4 motor
30 activity.

Previous work had suggested that MKLP1 may exist as a tetramer containing 4 motor domains (Chui et al., 2000; Kuriyama et al., 1994). If the two dimers were arranged in an antiparallel orientation, this arrangement could easily explain how this complex could crosslink antiparallel microtubules. However, the data obtained in the present invention are not consistent with this proposed structure. The complex that has been characterized appears to contain two kinesin motors and two RhoGAP molecules. Since most kinesin motors are dimers in which both catalytic cores interact with a single microtubule protofilament, the architecture that is described here can not easily explain how microtubule bundling is achieved. If the two kinesin subunits of centralspindlin bind to the same microtubule protofilament, then how might microtubule crosslinking occur? At this point, at least three possible mechanisms can be envisaged (fig. 18).

The first possibility is that there is an additional microtubule binding site elsewhere in the CYK-4/ZEN-4 complex. No additional binding site has been identified yet in CYK-4 nor has MKLP1, ZEN-4, or Pav been found to have an additional microtubule binding site. However, it has been shown, and was confirmed by the inventors, that MKLP1 interacts differently with microtubules than most kinesin-like proteins. Specifically, ATP is usually sufficient to elute most kinesins from microtubules, but in the case of MKLP1, both ATP and high salt are required (Kuriyama et al., 1994; Nislow et al., 1992). Thus it is possible that MKLP1 interacts with two microtubules, one by the motor domain and another via a different interaction surface.

Consistent with this possibility is the finding that Rab6KIFL kinesin, which is quite similar to MKLP1 in primary structure as well as in its localization and proposed function (Fontijn et al., 2001; Hill et al., 2000), has been reported to contain a second microtubule binding activity in the C-terminal half of the molecule (Echard et al., 1998). However this possibility does not explain why CYK-4 is required for central spindle assembly.

The second alternative is that MKLP1 forms higher order structures and that the tetramer that has been characterized in the present invention is merely a building block. This possibility gains some support from localization studies. In both *C. elegans* and in mammalian cells, ring-like structures are found that are termed division remnants that persist in the cell cortex after division. These remnants appear to be large aggregates of centralspindlin which are not in direct association with microtubules. Higher order oligomers could potentially form in early anaphase and promote microtubule bundling.

The third possibility is that, unlike most N-terminal kinesins, the two catalytic cores of the kinesin subunits in the centralspindlin complex could bind to different microtubules. This would not be without precedent in that the KIN-N KIF1A moves processively along a microtubule using a single head (Okada and Hirokawa, 2000). The association of the two catalytic cores of MKLP1 with different microtubules is made feasible by the fact that the linker region between the catalytic core and the coiled coil domain is much

longer than that present in most N-terminal kinesins. Perhaps CYK-4 ensures that the two motor domains are oriented in such a way to bind to antiparallel microtubules (fig 18). Structural analysis and
5 biochemical reconstitution of centralspindlin from purified components will allow further insight into mechanism of antiparallel microtubule bundling.

One critical question, which has not been rigorously explored to date is the function of the central spindle
10 during the process of cytokinesis. The obtained data do not shed extensive new light on this question. However, it may be noted that the RhoGAP domain of CYK-4 is highly conserved at the primary sequence level. Interestingly CYK-4 is significantly less
15 active at inducing GTP hydrolysis by RhoA as compared to Rac and Cdc42 (Jantsch-Plunger et al., 2000), and this feature of CYK-4 is conserved in HsCYK-4 (Toure et al., 1998). It has been proposed by the inventors that one function of the central spindle is to concentrate
20 CYK-4 to a position in the cell where it could promote inactivation of RhoA at a late stage in cytokinesis. Consistent with this model, inactivation of RhoA by RNAi does not prevent CYK-4 localization nor does it impair central spindle assembly. In addition, the
25 RhoGAP domain seems to be required at some stage of cytokinesis, since overexpression of a HsCYK-4/MgcRacGAP bearing a mutation in the RhoGAP domain causes cytokinesis defects (Hirose et al., 2001). Further structure-function analysis of CYK-4 is
30 necessary to test this model further. However such analyses must take into consideration that CYK-4 is able to dimerize or oligomerize and therefore such

analysis is best done in the absence of endogenous CYK-4.

Although centralspindlin appears to be a simple tetrameric complex, there are several lines of evidence that suggest that it may be subject to regulation by at least two different kinases. Genetic analysis of Polo kinase in *Drosophila* indicates Pav localization is Polo dependent (Carmenta et al., 1998) and moreover a biochemical interaction between Polo and MKLP1 orthologs have been shown in extracts from both mammalian cell and *Drosophila* embryos (Adams et al., 1998; Lee et al., 1995). In addition, the Aurora-B/Incenp complex is also required for the stable localization of centralspindlin in *C. elegans* embryos (Kaitna et al., 2000; Schumacher et al., 1998; Severson et al., 2000) and an in vitro biochemical interaction has been detected between Aurora-B (AIR-2) and ZEN-4 (Severson et al., 2000).

A stable association between Polo kinase or Aurora-B kinase with centralspindlin has been observed. Reconstitution of central spindle assembly in vitro will allow the role of these and other regulators of centralspindlin to be dissected.

In summary, the experiments of the present invention comprise an initial phenotypic, molecular, biochemical, and cell biological analysis of the *cyk-4* gene. Furthermore, they comprise an analysis of the CYK-4/ZEN-4 complex (centralspindlin) that consists of two molecules of each CYK-4 and ZEN-4.

- The experiments of the present invention (Examples 1-5) indicate that Cyk-4 is an active GTPase activating protein that is required for cytokinesis, likely by its ability to regulate the RhoA GTPase. Quite
- 5 surprisingly, one additional function of this protein is to promote assembly of the central spindle. Cyk-4 is a key molecule required for cytokinesis that regulates both the structure of the late mitotic spindle and the function of the contractile ring.
- 10 Thus, in a first aspect, the present invention generally relates to Cyk-4 proteins, which have been shown to be essential for this second microtubule dependent step and thus defines a functional link between the central spindle and the contractile ring.
- 15 In a preferred embodiment, the invention relates to a mammalian Cyk-4 polypeptide, in particular murine and human Cyk-4.
- In a first aspect, the invention relates to the murine Cyk-4 polypeptide with the amino acid sequence as set
- 20 forth in SEQ ID NO:4 or with the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3.
- In a preferred aspect, the invention relates to the
- 25 human Cyk-4 polypeptide with the amino acid sequence as set forth in SEQ ID NO:2 or with the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:1.

In a further aspect, the present invention relates to an isolated DNA molecule comprising a polynucleotide with the nucleotide sequence as set forth in SEQ ID NO:1 encoding human Cyk-4 polypeptide, or an isolated
5 DNA molecule encoding human Cyk-4 polypeptide comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:1.

In a further aspect, the present invention relates to
10 an isolated DNA molecule comprising a polynucleotide with the nucleotide sequence as set forth in SEQ ID NO:3 encoding murine Cyk-4 polypeptide, or an isolated DNA molecule encoding murine Cyk-4 polypeptide comprising a polynucleotide which hybridizes under
15 stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3.

By "stringent hybridization conditions" as used herein is meant overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (1X SSC = 150 mM
20 NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C, or equivalent conditions.

25 In the following, if not otherwise stated, the term "Cyk-4" refers to both the murine and the human Cyk-4.

Homologues of the subject Cyk-4 proteins also include versions of the polypeptide which are resistant to post-translation modification or which alter the activity of
30 the protein. The Cyk-4 polypeptide can comprise a full

length protein, such as represented in SEQ ID NO:2 or 4, or it can comprise a fragment or variant thereof.

Beside DNA molecules, the present invention relates to nucleic acid molecules in the form of RNA, such as mRNA. The DNA molecules include cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded.

Single-stranded DNA or RNA may be the coding strand, also known as the sense (or plus) strand, or it may be the non-coding strand, also referred to as the antisense (or minus) strand. The present invention also relates to preparations of double stranded Cyk-4 RNA or derivatives thereof that can be used to interfere with gene expression by ds-RNA mediated gene interference as described by Fire et al. 1998 and reviewed by Fire, 1999; Bosher and Labouesse, 2000; Sharp, 1999.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. Recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells, and those DNA molecules purified (partially or substantially) from a solution whether produced by recombinant DNA or synthetic chemistry techniques. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. However, it is intended that "isolated" as used herein does not include the Cyk-4 cDNA present in a cDNA library or in a preparation of purified or

isolated genomic DNA containing the Cyk-4 gene or a portion thereof in admixture with one or more other cDNA molecules or DNA fragments.

The nucleic acid molecules of the present invention
5 further include genetic constructs comprising one or more Cyk-4 DNA sequences operably linked to regulatory DNA sequences (which may be heterologous regulatory sequences), such as promoters or enhancers as described below, wherein upon expression of these DNA sequences
10 in host cells, preferably in bacterial, fungal (including yeast), plant or animal (including insect or mammalian) cells, one or more Cyk-4 polypeptides are produced. In such constructs, the regulatory sequences may be operably linked to a Cyk-4 polynucleotide
15 encoding mature Cyk-4 polypeptide or any of its variants, precursors, fragments or derivatives described herein, which may include one or more polynucleotides having a nucleic acid sequence that is complementary to substantially all or a portion of a
20 nucleic acid molecule having a nucleic acid sequence as shown in SEQ ID NO:1 or 3. As used herein, the terms "a portion" or "a fragment" of a nucleic acid molecule or a polypeptide means a segment of a polynucleotide or a polypeptide comprising at least 15, and more preferably
25 at least 20, contiguous nucleotides or amino acids of a reference polynucleotide or polypeptide (for example, the polynucleotide and polypeptide shown in SEQ ID NOs: 1 or 3, respectively, unless otherwise specifically defined below.)
30 Besides the DNA molecules having a nucleotide sequence corresponding to that depicted SEQ ID NO:1 or 3, the

invention also relates to DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the Cyk-4 mouse or human
5 polypeptides. Since the genetic code is well known in the art, it is routine for one of ordinary skill in the art to produce the degenerate variants described above without undue experimentation.

In addition, the invention relates to Cyk-4
10 polypeptides which have deviations from the sequence shown in SEQ ID NO:2 or 4, caused by the conservative exchange of amino acids, if they are Cyk-4 derivatives or fragments or peptides with the properties which are desirable for their use in therapy or in screening
15 assays, or isolated DNA molecules encoding such derivatives or fragments with a polynucleotide sequence varying in their sequence from SEQ ID NO:1 or 3.

Nucleic acid molecules of the present invention which
20 encode a Cyk-4 polypeptide or a derivative or fragment thereof may include, but are not limited to, those encoding the amino acid sequence of the polypeptide by itself, together with additional, non-coding sequences, including for example introns and non-coding 5' and 3'
25 sequences, such as the transcribed, untranslated regions (UTRs) or other 5' flanking sequences that may play a role in transcription (e.g., via providing ribosome- or transcription factor-binding sites), mRNA processing (e.g. splicing and polyadenylation signals)
30 and stability of mRNA; the coding sequence for the Cyk-4 polypeptide operably linked to a regulatory DNA

sequence, particularly a heterologous regulatory DNA sequence such as a promoter or enhancer; and the coding sequence for the *Cyk-4* polypeptide linked to one or more coding sequences which code for amino acids that

5 provide additional functionalities. Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain embodiments of this aspect of the invention,

10 the marker amino acid sequence may be a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described for instance in Gentz et al., 1989, hexa-histidine provides for

15 convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson et al., 1984. Yet another useful

20 marker peptide for facilitation of purification of *Cyk-4* is glutathione S-transferase (GST) encoded by the pGEX fusion vector (see, e.g., Winnacker, From Genes to Clones, New York: VCH Publishers, pp. 451-481 (1987)). As discussed below, other such fusion proteins include

25 the *Cyk-4* fused to immunoglobulin Fc at the N- or C-terminus.

A still further aspect of the present invention relates to antibodies and antibody preparations specifically reactive with an epitope of the *Cyk-4* polypeptide.

30 Polyclonal antibodies are conventionally obtained by immunising animals, particularly rabbits, by injecting

the antigen or fragments thereof and subsequently purifying the immunoglobulin.

Monoclonal anti-Cyk-4-antibodies may be obtained by standard procedures following the principle described
5 by Köhler and Milstein, 1975, by immunising animals, particularly mice, then immortalising antibody-producing cells from the immunised animals, e.g. by fusion with myeloma cells, and screening the supernatant of the hybridomas obtained by immunological
10 standard assays for monoclonal anti- Cyk-4 -antibodies. For therapeutic or diagnostic use in humans, these animal antibodies may optionally be chimerised in the conventional way (Neuberger et al., 1984, Boulianne et al., 1984) or humanised (Riechmann et al., 1988,
15 Graziano et al., 1995).

Cyk-4 specific antibodies can be used for both diagnostic and screening applications.

Cyk-4 specific antibodies are useful in clinical situations, where determining overexpression or
20 underexpression of CYK-4 has predictive value. Anti-CYK-4 antibodies can be used on tissue of cell specimens to evaluate the level of expression or changes in the subcellular localization in various disease states. In addition anti-CYK-4 antibodies can
25 be used to detect the presence of CYK-4 in solid-phase binding assays as described in the subsequent subsection related to finding compounds that affect the interaction of CYK-4 with other proteins.

In a further aspect the present invention provides
30 methods for identifying compounds capable of

modulating, in particular inhibiting cytokinesis by modulating, in particular inhibiting, the function of CYK-4 to promote GTP hydrolysis by Rho family GTPases.

5 This aspect of the invention is based on the finding that CYK-4 promotes GTP hydrolysis by Rho family GTPases. Due to the evolutionary conservation of the GTPase domain and the requirement for Rho in cytokinesis, it can be concluded that the ability of CYK-4 to promote GTP hydrolysis by Rho is crucial for
10 CYK-4 function.

The assay may be conducted on the basis of the so-called "GTP hydrolysis assay", which is a biochemical assay carried out according to standard protocols, as described, *inter alia*, by Self et al.,
15 1995, or by Settleman and Foster, 1995. By way of example, this assay may be carried out as follows: In a first step, the substrate is incubated with GTP that carries a radioactive label (e.g. the commercially available γ -³²P-GTP or an otherwise measurable, e.g. a
20 fluorescent label, as described, *inter alia*, by Hazlett et al., 1993, under conditions and for a period of time sufficient to allow saturation of GTP binding sites. Subsequently, CYK-4 is added, optionally at various concentrations of from 0 to eg 1 μ M; in the presence of
25 absence of test compounds. After the appropriate time (e.g. 5 min.) the amount of GTP that has been hydrolysed is determined.

Compounds that specifically affect the rate of CYK-4-dependent GTP hydrolysis by Rho are drug candidates

which may be further developed, e.g. in a first step, by structure-function analysis.

The assay of the invention may be performed in the high throughput format by automation of the reaction steps.

- 5 In this case, a great number of compounds, e.g. from compound or natural product libraries, are applied to microtiter plates containing the reagents for the binding reaction. After the time required for GTP saturation in the control reaction (absence of test
10 compound), the reaction solution may be filtered through a protein binding matrix, e.g. nitrocellulose, that is arranged in the same geometrical pattern as the original microtiter plate where the reaction took place, and the radioactivity retained in the filters,
15 or the amount of liberated free phosphate (or other label as appropriate) is quantified.

- To simplify the assay, the Rho protein may be immobilized on a solid matrix, either via a tag that allows for binding to a suitably modified solid
20 support, e.g. by using a biotinylated Rho protein and a streptavidin-coated microtiter plate, the solid matrix may also be in the form of beads. In the above-described assays that measure GTP hydrolysis, the Rho protein is preferably human.

- 25 By way of example, the screening assay may be conducted as follows: A member of the Rho family GTPases is immobilized on a solid support, e.g. a SPA (proximity scintillation assay bead) using either an epitope tag such as His6 or other similar tags. The Rho protein is
30 loaded with radioactive ^{32}P -gamma-GTP in the presence of

EDTA. Magnesium is added and the amount of label retained on the beads is measured by scintillation counting. Cyk-4 is added in the absence or presence of test compounds, incubated for various times at which point the amount of radioactivity or other suitable label remaining associated with the beads measured. Other embodiments of the assay include using alternative labels such as fluorescent GTP or other radioisotopes and using alternative measures of GTP hydrolysis such as measuring the amount of free ^{32}P released, as described above.

Alternatively to using the full-length Rho protein, a truncated version may be used, as long the GTPase activity is maintained, e.g. a fragment that lacks the critical cysteine residue in the CaaX box at the C-terminus that is required for membrane targetting.

A number of Rho proteins are available that are suitable for use in a GTP hydrolysis assay as a substrate, they have been cloned from various species. Preferably, they are employed as recombinant proteins.

Examples for Rho substrates that may be employed are human RhoA (Swiss Prot Primary Accession Number P06749), human RhoB (Swiss Prot Primary Accession Number P01121), human RhoC (Swiss Prot Primary Accession Number P08134), human RAC1 (Swiss Prot Primary Accession Number P15154), human RAC2 (Swiss Prot Primary Accession Number P15153), human RAC3 (Swiss Prot Primary Accession Number O14658) and human GB25 (Swiss Prot Primary Accession Number P21181).

An example for a co-factor which may be present in the assay reaction is polo kinase, e.g. the murine (Acc. No. NP 035251) or the human (Acc. No. NP 005021) polo-like kinase homolog.

- 5 Based on the sequence information, the Rho substrates and the potentially present co-factor(s) can be produced recombinantly in suitable host cells, e.g. in *E. coli* or by means of baculovirus in insect cells, according to standard methods.
- 10 Compounds that have the ability to prevent CYK-4 stimulated GTP hydrolysis by Rho family GTPases are then tested for specificity in a similar assay format using other proteins that have Rho family GAP domains, e.g. p190 or RhoGAP (Lamarche and Hall, 1994).
- 15 According to another aspect, the screening method of the invention is based on the biochemical interaction of CYK-4 and members of the MKLP1 subfamily of the kinesin-like protein superfamily.

- The function of CYK-4 in cytokinesis is intimately tied
- 20 to its ability to interact with MKLP1, a kinesin like protein that is also required for central spindle assembly. Therefore compounds that inhibit this interaction are expected to prevent cytokinesis and to inhibit cell proliferation. In *C.elegans* extracts,
 - 25 CYK-4 and CeMKLP1 are present in a protein complex and can be co-immunoprecipitated. The nature of this protein interaction can be further characterized, in particular by determining whether the interaction is direct or whether it requires additional proteins. In
 - 30 addition, the protein domains required for this

interaction can be mapped. Examples for members of the MLKP1 family are CeM03D4.1b (*C. elegans*; GenBank ID U61955, Protein ID 1397342) and HsMKLP1 (human; GenBank ID X67155; SwissProt Q02241).

- 5 The domains required for the interaction of Cyk-4 and MKLP1 can be identified using recombinant protein fragments of Cyk-4 and MKLP1. For example, agarose beads loaded with GST fusion proteins of Cyk-4 (or another fusion partner, e.g. maltose binding protein, etc.) are incubated with full-length or truncated fragments of MKLP1, the beads are sedimented and the ability of fragments of Cyk-4 to interact with MKLP1, or fragments thereof, are assessed by SDS-page. Alternatively, MKLP1 fragments can be immobilized and assessed for their ability to interact with full length Cyk-4 or fragments thereof. Alternatively, the yeast two hybrid assay can be used to measure the interaction of Cyk-4 protein fragments and MKLP1 protein fragments using standard techniques.
- 15
- 20 A minimal set of proteins necessary to form a stable complex, i.e. Cyk-4, MLKP1, and potentially additional protein co-factors are produced in recombinant form and a screening assay is conducted, preferably in the high throughput format, that measures the ability of test compounds to inhibit this interaction. Additional factors that may be required for the interaction can be identified in a biochemical complementation assay or a two hybrid assay. Since it is known that polo kinase forms a complex with Cyk-4, this protein is one of the potential co-factors required for the interaction with Cyk-4.
- 25
- 30

In an embodiment of the invention, the domain of Cyk-4 that interacts with MKLP1 (i.e. the N terminal Cyk-4 region containing at least amino acids 1-120), or the whole Cyk-4 protein, is immobilized on a solid support, as described below. The domain of MKLP1 that interacts with CYK-4 (i.e. the central MKLP1 domain containing at least amino acids 503-603) or the whole MLKP1 protein that is modified with a suitable label to allow for rapid detection (i.e. radiolabelled, fluorescently labeled, hapten labelled etc., as described below) is incubated in the presence or absence of the test compounds.

After an incubation period that allows for interaction of the proteins, e.g. for about 20 minutes at 25°C, the amount of MKLP1 bound to the immobilized CYK-4 is measured by use of the label outlined above or by the use of suitable antibodies in an ELISA type assay. The assay may also be setup in the reverse, e.g. with Cyk-4 being labeled or with MKLP1 immobilized, or by performing the binding reaction in solution and then capturing one of the components on a solid support and measuring the amount of the other component that is co-immobilized.

According to another aspect, the invention relates to a screening method that takes advantage of the biochemical multimerization of CYK-4 on the one hand and the biochemical multimerization of the MKLP1 subfamily of the kinesin-like protein superfamily on the other hand. This approach is based on the finding that the domain of CYK-4 that is required for interaction with MKLP1 contains a coiled-coil domain

that mediates self association. Likewise the domain of MKLP1 that is required to interaction with CYK-4 contains a coiled-coil domain that mediates self association.

- 5 Since the ability of these proteins to self association is expected to be required for their function in vivo, compounds that inhibit the self association of either one or both of the interaction partners Cyk-4 and MKLP1 are candidates for perturbing cytokinesis.
- 10 In an embodiment of the invention that is based on determining whether a test compound has the ability to interfere with the self association of Cyk-4, the N terminal domain of CYK-4 (containing at least amino acids 1-120), or the whole Cyk-4 protein, is
- 15 immobilized on a solid support, either directly or through a tag. (Suitable tags are commercially available, e.g. the FLAG, HA, MYC, HIS tag, etc.). Examples for solid supports useful in the present invention are commercially available immunobeads or
- 20 immunoplates, e.g. 96- well immunoplates, or microchips, which are coated with an antibody directed against one of the above-listed tags fused to the interacting protein.

- A second CYK-4 fragment consisting of or containing the
- 25 N terminal domain of CYK-4, i.e. amino acids 1-120, or the whole Cyk-4 protein is modified with a suitable label that allows for rapid detection. (The immobilized and the labeled Cyk-4 protein (fragments) may be identical or different.)

Examples for suitable labels are commercially available radioactive or fluorescence labels, hapten labels, or peptide labels, e.g. Europium or the Green Fluorescent Protein (GFP), an enzyme label, e.g. luciferase,
5 alkaline phosphatase etc.)

The labeled Cyk-4 (fragment) is incubated with the immobilized CYK-4 in the presence or absence of the test compounds.

After an incubation period that allows for interaction
10 of the proteins, e.g. for ca. 20 min, the amount of CYK-4 bound to the immobilized CYK-4 is determined by measuring the signal of the label or by the use of suitable antibodies in an ELISA type assay.

15 A further embodiment of the invention, the screening method is based on determining whether a test compound has the ability to interfere with the self association of MKLP1. In this embodiment, the central domain of MKLP1 (containing at least amino acids 503-603) or the
20 whole MKLP1 protein, is immobilized on a solid support.

A second MKLP1 fragment, consisting of or containing the above-defined central domain of MKLP1, or the whole MKLP1 protein is modified with a suitable label that allows for rapid detection. (The immobilized and the
25 labeled MKLP1 protein (fragments) may be identical or different).

The assay for identifying inhibitors of MKLP1 self assembly may be conducted in the the same way as

described above for identifying inhibitors of Cyk-4 self assembly.

Due to their ability of inhibiting the function of Cyk-4 to promote GTP hydrolysis by Rho family GTPase, of interfering with the interaction between Cyk-4 and MKLP1, or by interfering with self assembly of Cyk-4 and/or MKLP1, respectively, compounds identified in the above screens have the potential to perturb cytokinesis. In tumor cells, this effect may result in a decrease or a stop of tumor growth. In addition, inhibition of cytokinesis may cause the activation of a cell cycle arrest check point that will trigger apoptosis of the tumor cells.

To further evaluate the potential of the compounds as drugs, the candidate compounds can be assayed for their effect on cytokinesis and other cellular processes in tissue culture of normal or transformed cells. To test the inhibition of tumor cell proliferation, primary human tumor cells, are incubated with the compound identified in the screen and the inhibition of tumor cell proliferation is tested by conventional methods, e.g. bromo-desoxy-uridine or ^3H thymidine incorporation.

Compounds that exhibit an anti-proliferative effect in these assays may be further tested in tumor animal models and used for the therapy of tumors.

Thus, in a further aspect, the invention relates to compounds identified in the above screens for the therapy of tumors and any other situation in which cell overproliferation is observed.

Description of the figures.

Figure 1:

cyk-4(t1689ts) mutant embryos fail to complete
5 cytokinesis.

Figure 2:

cyk-4 mutant embryos produce deeply ingressing cleavage
furrows, but do not form a prominent central spindle.

Figure 3:

10 Positional cloning of the *cyk-4* locus.

Figure 4:

cyk-4(RNAi) causes disorganization of the proximal
gonad and affects formation of the central spindle.

Figure 5:

15 CYK-4 enhances GTP hydrolysis by Rho, Rac, and Cdc42.

Figure 6:

rho(RNAi) causes cytokinesis defects and *cdc42(RNAi)*
causes defects in spindle positioning.

Figure 7:

20 CYK-4 localizes to the central spindle and division
remnants.

Figure 8:

Time lapse analysis of CYK-4:GFP.

Figure 9:

CYK-4 and ZEN-4/CeMKLP1 colocalize and are
5 interdependent for their localization.

Figure 10:

Model for the function of CYK-4 in central spindle
formation and cytokinesis.

Figure 11:

10 RhoA does not have a role in the formation of central
spindle

Figure 12:

CYK-4 and ZEN-4 associate in vivo and in vitro

Figure 13:

15 The N- terminus of CYK-4 is necessary and sufficient to
bind to ZEN-4

Figure 14:

The central region of ZEN-4 is necessary and sufficient
to bind to CYK-4

20 Figure 15:

A complex of CYK-4 and ZEN-4 is required for function
in vivo

Figure 16:

Self-association of CYK-4 and ZEN-4

Figure 17:

A complex of MKLP-1 and HsCYK-4 in HeLa cells

5 Figure 18:

Centralspindlin and its role in the formation of
central spindle

10 In the Examples 1 - 5, if not otherwise stated, the
following materials and methods were used:

a) Strains and alleles

The *cyk-4(t1689ts)* allele was identified in a search
for maternal effect lethal mutations on chromosome III
15 (see (Gönczy et al., 1999b) for details). The strains
DR104, BW1535, BW1369, and RW7000 were obtained from
the CGC. The strain EU699 containing the
zen-4(or153ts) allele was described by Severson, et
al., 2000. The end points of the deficiency tDf10
20 (Heinke Schnabel, unpublished data) are not molecularly
defined, but it uncovers *cyk-4*, *lit-1*, and *bli-5* and it
does not uncover *unc-64*.

b) Antisera

Cyk-4 specific antisera were produced in rabbits using a His6-Cyk-4 fusion protein as immunogen (containing amino acids 407-613 of *cyk-4*). A GST-Cyk-4 fusion
 5 containing amino acids 407-681 of Cyk-4 was coupled to a Hi-Trap NHS resin (Amersham-Pharmacia) and used to affinity purify anti-Cyk-4 antibodies which were used at a final concentration of 1:300. The antibodies used for the studies are specific for Cyk-4 since the
 10 staining can be blocked with antigen, a similar pattern is observed when anti-GFP antibodies are used to detect a Cyk-4:GFP fusion construct, and the staining pattern is disrupted in *cyk-4* mutant embryos. The rat monoclonal YOL 1/34 anti-tubulin antibody was used at a
 15 dilution of 1:200-500. Anti-GFP antibodies (Roche) were used at a dilution of 1:500. Antisera specific for Zen-4/MKlp1 was generously provided by Bill Saxton and Susan Strome (Univ. of Indiana) and used at a dilution of 1:4000. Antisera specific for Air-2 was
 20 generously provided by Andy Golden (NIH) and used at a dilution of 1:1000.

c) Genetic mapping of *Cyk-4*

The *cyk-4* locus maps under the deficiency tDf6 which
 25 deletes a large fraction of the distal right arm of LGIII. Recombination mapping using *unc-32(e189)* *cyk-4(t1689ts)/dpy-18(e364)* *unc-25(e156)* placed *cyk-4* distal to (or very close to) *unc-25* (23/23 Dpy non Unc's carried the *cyk-4* mutation). Recombination
 30 between *dpy-18 cyk-4/RW7000* which carries several Tc1

elements including one on the cosmid F14F7 gave rise to 45 Dpy non-Ts animals, 3 of which lacked the TC1 insertion on F14F7, indicating that the *cyk-4* gene is distal to this cosmid. Crosses to strains carrying the deficiencies *ctDf3*, *ctDf2*, *tDf10* revealed that *cyk-4(t1689ts)* is not uncovered by *ctDf3* and is uncovered by both *ctDf2* and *tDf10*. Since *tDf10* does not uncover *unc-64*, *cyk-4(t1689ts)* must be distal to *unc-64*.

10

d) Time Lapse recordings

Time lapse nomarski imaging was performed as described previously (Jantsch-Plunger and Glotzer, 1999). Time lapse imaging of *Cyk-4::GFP* was performed on a Zeiss Axiovert microscope using a 100X/1.3 neofluar objective. The illumination source, an Atto-arc HBO-103, was reduced to 50% intensity. An intensified cooled CCD camera (GenIV pentamax, Princeton Instruments) was used for image acquisition. The camera and other electronics were controlled with MetaMorph software (Universal Imaging). Typical acquisition times were 40-80 msec. Each 10 seconds, 4-5 fluorescent images were acquired at different focal planes and a nomarski image was acquired. The fluorescent images were projected onto a single frame using the maximum intensity from the stack of images. Under these conditions, embryos could be filmed for more than one hour without affecting the cell cycle timing or pattern of cell divisions.

30

e) Rescue Experiments

To identify the *cyk-4* gene in this region cosmid DNA (from stocks kindly provided by Alan Coulson, Sanger Center) was coinjected with the *rol-6(su1006)* dominant marker (Mello et al., 1991) into the gonad of *unc-32* *cyk-4/qC1* worms. Heterozygous F1 hermaphrodites that carried the *rol-6* dominant marker were cloned to individual plates at 25°C and the presence of Unc progeny, indicating zygotic rescue of the *cyk-4* mutation, was assessed. Individual *unc-32*, *cyk-4* worms carrying the extrachromosomal arrays were cloned to individual plates to assess the extent of germline rescue. The *cyk-4* genomic rescue construct MP17, contains a 4.9 kb genomic *XbaI* fragment excised from K08E3 and inserted into pBS-KS+.

f) RNA interference

Approximately 500 bp of DNA corresponding to predicted coding regions of Rho (Y51H4A.B), Rac-1 (C09G12.8B), Cdc42 (R07G3.1), F22E12.2, Y32F6B.3, K08D3.9, K08E3.2, K08E3.3, K08E3.4, K08E3.6, K08E3.7, K08E3.8 were amplified by PCR and cloned into pGEM-T (Promega). Double stranded RNA was transcribed (Ambion) and annealed, and injected into the gonads of wildtype N2 hermaphrodites as described (Fire et al., 1998).

g) Production of Cyk-4:GFP transgenes

The GFP cassette from vector pPD119.16 was excised with BspLUIII and inserted into the unique NcoI site of MP17 (see above). This construct was linearized with XbaI, and complex arrays containing linearized genomic DNA and linearized rol-6(sul006) DNA were mixed in a ratio of 1:100:1 and injected into *unc-32(e189)* *cyk-4(t1689ts)/qC1* hermaphrodites. Rolling F1 heterozygotes were singled out at 25°C and rolling Unc F2 animals were picked. A line MG110, was obtained that gave stable rescue of the *cyk-4(t1689ts)* mutation.

The structure of the *cyk-4* gene was established by analysis of a large number of EST sequences available in the sequence databases at the Sanger Center and the National Institute of Genetics and by sequencing the clones yk63D6 and yk104g12 (kindly provided by Yuji Kohara). The structure of the gene is identical to the structure predicted by the *C. elegans* genome consortium.

20

h) Immunolocalization

Immunolocalization studies were performed as previously described (Jantsch-Plunger and Glotzer, 1999). Briefly, gravid hermaphrodites were placed on aminopropyl-silane treated slides, a coverslip was added, and sufficient pressure to extrude the embryos was applied. The slide was placed into liquid nitrogen. The coverslip was removed while the sample was still frozen, the preparation fixed with -20°C

methanol and then antibody staining was performed according to standard procedures.

i) Biochemical analysis of Cyk-4

5 The coding regions of Rho, Rac, and Cdc42 were PCR amplified and cloned into pET28b with an C-terminal polyhistidine tag. The GTPases were expressed at 25°C and purified using Ni²⁺-NTA-agarose (QIAGEN). Proteins were dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM
10 MgCl₂ and quick frozen. The GAP domain of Cyk-4 (amino acids 407-681) was cloned into pGEX4T-1. Proteins were expressed at 25°C and purified using GSH-agarose (Sigma). Proteins were dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT and quick frozen.
15 To assess GAP activity, 15 pmol of the GTPases were loaded with 1 pmol ³²P-α-GTP in 20 mM Tris pH 7.6, 4 mM EDTA, 25 mM NaCl, 1 mM DTT, 1 mM ATP, 0.1 mg/ml BSA at room temperature. The sample was place on ice and MgCl₂ was added to 17 mM. GST-Cyk-4-GAP was added at the
20 indicated concentrations and, at intervals, samples were taken by dilution into 2% SDS, 20 mM EDTA. Aliquots were spotted onto TLC plates (PEI-cellulose, Machery-Nagel) and developed in 1M LiCl. The plates were dried and exposed using a Storm Phosphoimager
25 (Molecular Dynamics) and the data analysis was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health; <http://rsb.info.nih.gov/nih-image/>).

Example 1

cyk-4 mutants initiate, but do not complete, cytokinesis

The *cyk-4(t1689ts)* allele was isolated in a screen for maternal effect embryonic lethal mutations on chromosome III (Gönczy et al., 1999b). The *cyk-4* locus is defined by a single, temperature sensitive (ts), allele. The phenotype of embryos derived from homozygous *cyk-4* hermaphrodites at the restrictive temperature (hereafter referred to as *cyk-4* mutant embryos) during the first division is shown in figure 1B. *cyk-4* mutant embryos appear normal until cytokinesis, except that defects in polar body extrusion are frequently observed (data not shown). The first cleavage furrow forms at the correct time and place, it ingresses extensively, but, invariably, it regresses. Quantitation of the extent of furrow ingression in *cyk-4* mutant embryos reveals that, on average, furrows ingress to $73 \pm 13\%$ ($n=14$) of the egg diameter. A multipolar spindle develops in the second cell cycle and the process of furrow ingression and regression occurs again. This pattern is repeated until the embryos become grossly disorganized. (Figure 1 shows that *cyk-4(t1689ts)* mutant embryos fail to complete cytokinesis. Wildtype embryos (A) and embryos from *cyk-4* mutant hermaphrodites (B) were dissected from young adults, mounted on agarose pads and observed by time-lapse nomarski microscopy. The ingressing cleavage furrow is indicated (arrows). 10 μ m scale bars.)

Although the majority of cell divisions in worm development occur early in embryogenesis, cells of the germline and many cells in the nervous system are produced during post embryonic development (Sulston and
5 Horvitz, 1977). A number of mutations in genes required for cell division cause worms to become sterile and uncoordinated (O'Connell et al., 1998; Woollard and Hodgkin, 1999). To determine if *cyk-4* is required post embryonically, temperature shift experiments with worms
10 homozygous for the *cyk-4(t1689ts)* allele were performed (table 1). Homozygous animals grown at 16°C are viable and fertile. Homozygous *cyk-4* animals shifted to 25°C at the L4 stage produce embryos with the cytokinesis defects described above. Homozygous animals shifted to
15 25°C at earlier stages either fail to hatch or become sterile and uncoordinated, depending on the time of the temperature shift. Thus *cyk-4* is required postembryonically, perhaps due to its role in cell division. Unexpectedly, animals shifted at the L2/L3
20 stages become highly uncoordinated adults, even though most motor neurons would be expected to have completed all their divisions at the time of the shift. This may suggest that CYK-4 has additional roles besides its role in cell division. However, not out the
25 possibility cannot be ruled out that some of the cells in the ventral nerve cord divide later in *cyk-4* mutants.

To investigate why *cyk-4* mutant embryos fail to complete cytokinesis, actin and tubulin were localized
30 in wild-type and *cyk-4* mutant embryos. Both wild-type and mutant embryos contained deeply ingressing cleavage furrows that stain with an anti-actin antibody; the

results are shown in figure 2. Figure 2A,B shows that *cyk-4* mutant embryos produce deeply ingressing cleavage furrows, but do not form a prominent central spindle. Wild type (A, C) and *cyk-4* mutant embryos (B,D) were
5 fixed and stained for actin (green) and DNA (blue) (A,B); tubulin (green) and DNA (blue) (C,D). 10 μ m scale bars.

The metaphase spindles of *cyk-4* mutant embryos appeared normal. However, spindle morphology during early
10 anaphase was significantly different in mutant embryos as compared to wild type. In wild-type embryos (20/20) prominent microtubule bundles form between the separating masses of chromatin, forming the central spindle (figure 2C). In *cyk-4* mutant embryos (9/10)
15 such bundles were largely reduced and disorganized (figure 2D). It is concluded that CYK-4 is required for the organization of the central spindle during anaphase. Since the central spindle is required for cytokinesis, it is possible that the *cyk-4* mutant
20 embryos fail to complete cytokinesis because they fail to assemble the central spindle.

Example 2

Cloning of the *cyk-4* gene

25 To investigate the molecular basis for the phenotypes described above, it was first sought to map the *cyk-4* locus and clone the affected gene (see methods for details). The *cyk-4* gene maps distal to *unc-64* on the extreme right arm of LG III. Figure 3 shows the

of LGIII showing the positions of various loci and the extent of the deficiencies. *cyk-4* is uncovered by *tDf10*, *tDf6*, and *ctDf2*, but not *ctDf3*. (B) An enlargement of the physical map from the *unc-64* locus until the end of LGIII. The ability of various cosmid pools to rescue *cyk-4* is indicated. (C) A map of the predicted genes on K08E3. All the predicted genes except for K08E3.1 and K08E3.5 were inactivated by RNAi and only RNAi of K08E3.6 produced multinucleate embryos. (D) A schematic representation of the domain structure of CYK-4 and its human ortholog. The position of the point mutation identified in *cyk-4(t1689ts)* is indicated.

15 The *cyk-4* gene was then identified by functional rescue of the zygotic requirement for *cyk-4* using pools of cosmids. A pool of three cosmids (ZK520, W06F12, and K08E3) allowed *cyk-4* homozygotes to hatch and develop to adulthood at 25°C. These cosmids were injected individually and cosmid K08E3 contained rescuing activity. Further subcloning revealed that a 4.9 kb genomic fragment, predicted to contain the complete K08E3.6 gene and no other intact gene, could rescue the *cyk-4* zygotic and germline phenotypes. Finally, the coding region was amplified from DNA derived from *cyk-4(t1689)* homozygotes and sequenced and a single point mutation was identified that differed from the sequence provided by the genome project; this mutation was not observed in another line derived from the same parental strain. These data show that the defect in *cyk-4(t1689ts)* embryos is due to a point mutation in the K08E3.6 gene.

The predicted protein product of the *cyk-4* gene has a C-terminal domain that contains the consensus motifs of GTPase activating proteins for Rho family GTPases (figure 3). Adjacent to the C-terminal GAP domain is a
5 C1 domain that is predicted to bind to diacylglycerol or phorbol esters. At the amino terminus of the protein is a 90 amino acid region predicted to form a coiled-coil domain. The S15L point mutation found in *cyk-4(t1689ts)* , is located just amino terminal to the
10 coiled-coil domain. Human and mouse proteins with structural similarities to *cyk-4* have been described (Toure et al., 1998; Wooltorton et al., 1999). These genes are expressed in a variety of proliferating tissues. In addition, the *Drosophila* sequence database
15 contains an entry (ascension no. AC005977 (CLOT 94)) that, together with CYK-4 and the previously mentioned human gene, share a common structure consisting of approximately 650 amino acids, an NH₂-terminal coiled-coil domain and a conserved C-terminus containing C1
20 and GAP domains. The structural conservation of CYK-4 suggests that its function is conserved among metazoans.

In the present invention, the the human and mouse cDNA's were identified by searching the DNA databases
25 with the *C.elegans* CYK-4 protein sequence.

Since the *cyk-4(t1689ts)* mutation may contain residual activity RNA-mediated interference (RNAi) was used to deplete embryos of CYK-4 protein.

Figure 4 shows that *cyk-4(RNAi)* causes disorganization
30 of the proximal gonad and affects formation of the

central spindle. Young adults were injected with *cyk-4* dsRNA and the injected animals were analyzed 30 hours after injection (B). The gonad of an uninjected worm is shown for comparison (A). Wild-type embryos (C) and
5 embryos from *cyk-4(RNAi)* injected animals (D) were fixed and stained for tubulin. 10 μ m scale bars.

RNAi of the predicted open reading frame K08E3.6 generated multinucleate embryos which exhibited a similar phenotype to that of the *cyk-4* mutant,
10 including loss of the central spindle (figure 4 D) and incomplete cytokinesis (not shown). Interestingly, the gonads of *cyk-4(RNAi)* animals become disorganized 30 hours post-injection (figure 4 B) and irregularly sized embryos are produced, suggesting that CYK-4 acts
15 not only during embryonic and zygotic development, but also in the female germline.

Example 3

Biochemical activity of the Cyk-4 GAP domain

20 The presence of a Rho-family GAP domain suggests that CYK-4 may regulate one or more GTPases of Rho branch of the GTPase superfamily. To determine whether CYK-4 is active as a GAP and whether its GAP activity is restricted to particular members of the Rho subfamily,
25 a recombinant fusion protein containing GST and the CYK-4 GAP domain was prepared and GTP hydrolysis assays were performed with recombinant *C. elegans* Rho, Rac, and Cdc42. The GAP domain of CYK-4 promotes GTP hydrolysis by all three tested GTPases. Figure 5 shows

that CYK-4 enhances GTP hydrolysis by Rho, Rac, and Cdc42. The GTPases were preloaded with ^{32}P - α -GTP and then GST-CYK-4-GAP or GST was added at the indicated concentration. Samples were taken at two minute
5 intervals, the labeled nucleotide was resolved by thin layer chromatography, the fraction of GTP and GDP was quantitated. This graph shows the fraction of GTP hydrolyzed at the 2 minute time point as a function of CYK-4 concentration. These data are representative of
10 at least three independent experiments.

However kinetic differences were observed. At the conditions used in the assays it was found that the CYK-4 GAP domain is more active towards Rac and Cdc42 than towards Rho. The human ortholog has a similar
15 activity profile *in vitro* (Toure et al., 1998). However, since CYK-4 has activity towards all three GTPases, these data are not sufficient to determine the *in vivo* target(s) of the CYK-4 GAP domain.

Next RNAi was used to determine which, if any, of the
20 Rho family GTPases are required for cytokinesis in the *C. elegans* embryo. RNAi experiments were performed with RhoA, Rac1, Cdc42 and three additional GTPases found in the genome that fall into the Rho subfamily. Approximately 90% of *Rho(RNAi)* embryos exhibit
25 cytokinesis defects in the first and/or second cell cycle (table 2 and figure 6).

Figure 6 shows that *rho(RNAi)* causes cytokinesis defects and *cdc42(RNAi)* causes defects in spindle positioning. Young adults were injected with the
30 indicated dsRNAs (see table 2) and the embryos produced

by the injected worms were analyzed by time lapse nomarski microscopy. Images from a wild-type (A), *rho(RNAi)* (B) and a *cdc42(RNAi)* (C) embryo are shown. 10 μ m scale bars.

- 5 In most embryos, furrow ingression was inhibited. Interestingly, central spindles assemble in *RhoA(RNAi)* embryos (data not shown). In contrast, 88% of *Cdc42(RNAi)* embryos complete cytokinesis normally. However, a distinct defect in the early embryo is
10 observed in 54% of *Cdc42(RNAi)* embryos; defects in spindle positioning are observed in P0 and/or P1. A minority (12%) of *Cdc42(RNAi)* embryos fail to initiate cytokinesis; in most cases, these embryos appear
15 osmotically swollen even when provided with osmotic support. *Rac(RNAi)* embryos hatch with high efficiency and did not exhibit a detectable phenotype in the early embryo. RNA interference experiments with the additional GTPases either alone or in combinations did not reveal any additional defects in the early embryo.
20 Thus *RhoA* is the only member of the *Rho* family that is clearly required for cytokinesis and is therefore likely to be the critical target for the CYK-4 GAP domain.

25 Example 4

The subcellular localization of Cyk-4

Next the subcellular localization of CYK-4 protein was determined. CYK-4 localization is cell cycle dependent

Figure 7 shows that CYK-4 localizes to the central spindle and division remnants. (A-F) Wild-type embryos were fixed and stained for CYK-4 (green), tubulin (red) and DNA (blue). The localization of CYK-4 to the central spindle (arrow) in a one cell embryo (D) and a two cell embryo (F) is shown. The localization of CYK-4 to the division remnant from the polar body (A), and between the AB and P1 blastomeres is indicated with arrows. (E). (G) An embryo from a line expressing CYK-4:GFP is stained with anti-GFP antibodies. The same structures are seen as with CYK-4 antibodies (arrow). (H) The intrinsic fluorescence of the gonad of a worm expressing CYK-4:GFP. CYK-4 is seen at the incomplete membranes of the syncytial gonad (arrow) and in oocyte nuclei. 10 μ m scale bars.

In interphase cells, CYK-4 is present in the cytoplasm and slightly concentrated in the nucleus. CYK-4 is also highly concentrated in a spot at the anterior of the embryo, DNA labeling reveals that this localization corresponds to the site of polar body extrusion. As embryos enter mitosis, CYK-4 protein concentrates around the mitotic spindle. In early anaphase, CYK-4 concentrates to the central spindle. As the cleavage furrow ingresses, CYK-4 becomes highly concentrated on the central spindle into a structure that often appears ring shaped (not shown). Upon completion of cytokinesis, CYK-4 staining persists at division remnants. CYK-4 organized in ring-like structures averaging 1.2 μ m in diameter are occasionally observed in the cytoplasm (not shown).

To determine if CYK-4 localizes to the central spindle prior to the onset of cleavage furrow ingression, the dynamics of CYK-4 localization in live embryos was investigated. To accomplish this goal a transgenic
5 line expressing a CYK-4:GFP fusion was generated and its localization followed by time lapse microscopy. The CYK-4:GFP fusion is partially functional since *cyk-4 xSEx1[cyk-4:GFP]* animals are viable and fertile at 25°C whereas the parental *cyk-4* strain is inviable
10 at 25°C. However, the fusion construct does not fully rescue the mutation, since about 40% of embryos produced by this line fail to hatch (table 1).

Low light level fluorescence microscopy was used to visualize CYK-4:GFP in living embryos. Embryos were
15 imaged using a multi-mode imaging system whereby a series of z-sections and a nomarski image were recorded every 10 seconds. The fluorescent images from each time point were projected to form a single image.

Figure 8 shows the time lapse analysis of CYK-4:GFP.
20 An embryo from a line expressing CYK-4:GFP was imaged using low light level microscopy. The central spindle localization of CYK-4 is observed prior to furrow ingression. 10 µm scale bars.

These recordings reveal that CYK-4 accumulates on the
25 central spindle prior to the initiation of furrowing (figure 8; 3:40). The CYK-4 that localizes to the central spindle becomes compressed into a bright spot which persists at the division remnant. The remnant persists for several cell cycles although instances
30 were observed whereby the remnant (sometimes from the

polar body) detaches from the cortex and is observed as a discrete spot in the cytoplasm. This detachment of CYK-4 from division remnants likely accounts for the CYK-4 rings seen in fixed specimens. It is concluded
5 that CYK-4 localization on the central spindle precedes furrow ingression.

Example 5

Cyk-4 and zen-4/CeMklp1 are functionally interdependent

10 There are remarkable similarities between CYK-4 and the kinesin-like protein ZEN-4/CeMKLP1 (Powers et al., 1998; Raich et al., 1998). *zen-4* mutant embryos also initiate, but fail to complete cytokinesis. They also fail to assemble a robust central spindle in early
15 anaphase. Furthermore, ZEN-4 localizes to the central spindle and persists at division remnants after completion of cytokinesis. To test whether these proteins functionally interact, it was first assessed whether CYK-4 and ZEN-4/CeMKLP1 co-localize.

20 Embryos expressing CYK-4:GFP were fixed and CYK-4 and ZEN-4 were localized simultaneously (using an anti-GFP antibody to detect CYK-4:GFP). The two proteins co-localize both on central spindle structures and on division remnants (Figure 9A-C). It was next
25 investigated if ZEN-4 localization requires functional CYK-4 protein. ZEN-4 staining of *cyk-4* mutant embryos reveals that ZEN-4 localization to the central spindle is absent (figure 9E), although staining of some microtubule bundles in the spindle midzone could be

detected using an AIR-2 antibody (figure 9G). Thus, recruitment of ZEN-4 to the central spindle is CYK-4 dependent.

Figure 9 shows an embryo expressing CYK-4:GFP stained
5 for GFP (A), ZEN-4/CeMKLP1 (B) and the merged image
(C). CYK-4 and ZEN-4/CeMKLP1 colocalize at division
remnants (arrow) and a central spindle (arrowheads)
structures. ZEN-4 localization to the central spindle
is CYK-4 dependent (arrow). Wild-type (D) and
10 *cyk-4(t1689ts)* embryos (E) were fixed and stained for
ZEN-4/CeMKLP1 (green), tubulin (red) and DNA (blue).
AIR-2 localization to the central spindle is CYK-4
independent (arrow). Wild-type (F) and *cyk-4(t1689ts)*
embryos (G) were fixed and stained for AIR-2 (green),
15 tubulin (red) and DNA (blue). CYK-4 maintenance to
division remnants (arrowheads) is ZEN-4/CeMKLP1
dependent. *zen-4(or153ts)* worms were maintained at
16°C and either fixed immediately (H) or shifted to
25°C for 18 minutes (I). Embryos were fixed and stained
20 for CYK-4 (green), tubulin (red) and DNA (blue). 10 µm
scale bars.

Next it was tested whether maintenance of ZEN-4 at
division remnants requires functional CYK-4. *cyk-4*
mutant embryos grown at 16°C were shifted to 25°C for
25 15 minutes prior to fixation and staining with anti-
ZEN-4 antibodies. The number of cells and number of
division remnants labeled with the anti-ZEN-4 antibody
were counted. Embryos maintained at the permissive
temperature had a large number of ZEN-4 staining
30 division remnants (table 3) whereas the embryos shifted
to the non-permissive temperature lacked defined

staining of division remnants. Thus both recruitment of ZEN-4 to the central spindle and its maintenance at division remnants is CYK-4 dependent. The reverse experiment was conducted with a temperature sensitive allele of *zen-4(or153ts)*. In this case CYK-4 staining at division remnants was observed in *zen-4* mutant embryos at the permissive temperature but this staining disappeared upon a brief shift to the non-permissive temperature (figure 9H,I and table 3). Thus maintenance of CYK-4 at division remnants is ZEN-4 dependent. It is concluded that CYK-4 and ZEN-4/CeMKLP1 colocalize and that the two proteins are interdependent for their localization.

Next, it was determined if embryos carrying mutations in both *cyk-4* and *zen-4* are distinguishable from the single mutants. Two strains were built, one strain was homozygous for *zen-4(or153ts)* and heterozygous for *cyk-4(t1689ts)* and the second strain was homozygous for *cyk-4(t1689ts)* and heterozygous for *zen-4(or153ts)*. Both strains were viable at 16°C, but they failed to produce doubly homozygous larvae. It was found that worms of genotype *unc-32(e189) cyk-4(t1689ts) /qC1 III, zen-4(or153ts)* laid a fraction of embryos that arrested during embryonic development, typically before the comma stage. Thus *cyk-4(t1689ts)* and *zen 4(or153ts)* are synthetically lethal.

In the subsequent Examples, the following Materials and Methods were used:

i) Worm Strains and Alleles

The following alleles were used: N2(Bristol), CB4856, *cyk-4(t1689ts)*, *unc-64*, *bli-6(scl6)*, *unc-24(e138)*,
5 *unc-44(e1260)*, *lag-1(q385)*, and *mIs11 IV*. Some strains were obtained from the *C. elegans* Genetics Center.

ii) Isolation and mapping of *cyk-4* suppressor alleles

Suppressors of the *cyk-4(t1689ts)* mutation were obtained by mutagenizing *cyk-4(t1689ts)* animals with
10 30-40 mM EMS or 0.5 mM ENU. P0 animals were allowed to self fertilize for two generations at the permissive temperature. When F2 animals reached early adulthood, the population was shifted 20°C and fertile animals were selected. Approximately 110,000 F1 genomes were
15 screened and 18 suppressor mutations, all but 2 being unambiguously independent, were isolated. While all of the suppressor strains were viable and fertile at 20°C, none were able to grow at 25°C, suggesting that none of them precisely reverted the original mutation. Several
20 intragenic suppressor mutations were identified. The ZEN-4 coding region of the remaining mutants were sequenced and 7 strains were found to contain substitutions within the CYK-4 binding region of ZEN-4 and one contained a substitution in the catalytic
25 domain. One suppressor strain, *xs82*, was characterized in detail. Suppressor activity was mapped using single nucleotide polymorphisms (SNPs) to the central region of chromosome IV (see methods). The ZEN-4 coding region was sequenced in this strain and a point mutation was

found that causes a substitution of glutamic acid for a lysine at position 502 (fig 15A).

iii) Cell Culture

HeLa cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin.

iv) Antibodies

Rabbit CYK-4 specific and ZEN-4 specific antisera were reported previously (Jantsch-Plunger et al., 2000). CYK-4 and ZEN-4 antibodies were affinity-purified with His6-CYK-4 (407-613) or His6-ZEN-4 (578-775) immobilized on NHS-Sepharose (Pharmacia).

MKLP-1 specific antisera and HsCYK-4 specific antisera were produced in rabbits and mice (Gramsch Laboratories, Schwabhausen, Germany) using C-terminal peptides (QLGPGYQHHAQPKRKKP and SKSKSATNLGRQGNFFASPMK, respectively) conjugated to keyhole limpet hemocyanin as immunogens. Rabbit antibodies were affinity purified using peptides immobilized on Poros epoxide resin.

v) Plasmids

A cDNA clone for ZEN-4 in pBluescript (yk391b3) which lacks 13 nts at 5' of the coding region which were introduced by PCR. The pCBD-TEV vector was constructed by inserting the sequences encoding the chitin-binding domain (CBD) and a TEV protease site into pET28a

(Novagen). ZEN-4 fragments were amplified by PCR and cloned into pCBD-TEV.

vi) In Vitro Binding Assay

Full length and fragments of CYK-4 and ZEN-4 were
5 expressed by in vitro transcription and translation
system with reticulocyte lysates using the TNT® Coupled
Reticulocyte Lysate Systems (Promega) or the
PROTEINscript™ II (Ambion) kits, typically in 20 µl
reactions. Full length proteins without tag were
10 expressed from the T3 promoter of pBluescript SK(-).
Fragments of CYK-4 and ZEN-4 tagged with chitin-binding
domain (CBD) at N-terminus were expressed from the T7
promoter of pET-CBD. In some experiments (Fig. 12C,
13C and 16), CYK-4 and ZEN-4 were co-expressed in the
15 same reactions. In other experiments, they were
separately expressed and mixed. Following incubation
at 20°C for 30 min in 100 µl buffer A (20 mM Hepes,
150 mM NaCl, 2 mM MgCl₂, 10 mM EDTA, 1 mM DTT, 1 mM
PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml
20 chymostatin) with 0.5% (w/v) Triton X-100, ZEN-4 or
CYK-4 proteins were immunoprecipitated with specific
antibodies, or affinity-purified by chitin beads. For
immunoprecipitation, 0.5 µg antibody was added to the
reactions and incubated on ice for 1 hr, followed by
25 incubation with 5 µl protein A-Sepharose beads at 4°C
for 1h. For CBD-affinity purification, 5 µl of chitin
beads (New England Biolabs) were added. In both cases,
the beads were washed three times with buffer A
containing 0.5% (w/v) Triton X-100. Proteins bound to
30 the beads were analyzed by SDS-PAGE followed by
autoradiography with PhosphoImager.

vii) Preparation of Cell Lysates

C. elegans embryos were prepared by bleaching a synchronous culture of adult worms grown on "egg plates" as described (Lewis and Fleming, 1995). The
5 embryos were washed with buffer A without Triton-X 100 and frozen with liquid nitrogen and kept at -80°C until use. Embryos were crushed by grinding in a mortar and pestle in liquid nitrogen.

HeLa cells were synchronised by treatment with 0.1
10 $\mu\text{g/ml}$ nocodazole for 16 h. Cells were washed twice with cold PBS, frozen by liquid nitrogen and kept -80°C until use. Cells were lysed and thawed by suspending into 10 volumes of buffer A with 0.5% (w/v) Triton X-100.

15 viii) Preparation of Microtubule-binding Fraction

Frozen pellet (0.5 ml) of nocodazole arrested M-phase cells was thawed and lysed in 5 ml ice-cold BRB80 (80 mM K-Pipes, 1 mM MgCl_2 , 1 mM EGTA, pH 6.8) with 0.1% (w/v) Triton X-100. The lysate was clarified by
20 centrifuging at $10,000 \times g$ for 15 min. The resulting supernatant was centrifuged at $25,000 \times g$ for 20 min at 4°C in S100AT6 rotor (Hitachi). Microtubules polymerized with glycerol and taxol was added to the supernatant. After incubating at 20°C for 20 min,
25 microtubules were pelleted by centrifuging at $25,000 \times g$ for 20 min at 20°C . The bound proteins were released from microtubules by incubation in 0.5 M NaCl, 5 mM ATP in BRB80 at 20°C for 20 min, followed by centrifugation at $25,000 \times g$ for 20 min at 20°C .

ix) Mass Spectrometry analysis

Hydrodynamics

Sedimentation coefficient was estimated by ultracentrifugation through 2 ml linear gradient of 5 to 20% (w/v) sucrose in buffer A without Triton X-100 using S55S rotor (Hitachi). 150 µl cell lysate or microtubule binding fraction was applied. Chicken ovalbumin (3.4 S), bovine gamma globulin (7.1 S), bovine catalase (11 S) and bovine thyroglobulin (19 S) were used as standards. The diffusion coefficient was estimated by gel filtration using Superdex 200 column (30 ml) in FPLC system (Pharmacia).

Immunoprecipitation and Western Blotting

Lysates (500 µl) were precleared with 50 µl protein A-Sepharose beads. For immunoprecipitation from worm embryos, 1 µg affinity-purified antitibody was added to the lysate. After incubation on ice for 1 hr, immunocomplex was recovered by incubation with 5 µl protein A-Sepharase (Pharmacia) at 4°C for 1 hr. For immunoprecipitation from HeLa cells, antibodies were covalently immobilized on protein A-beads (1 µg per 1 µl beads) with dimethyl pimelimidate. The precleared lysate was incubated antibody-beads at 4°C for 1 to 4 h. The beads were washed briefly with buffer A plus 0.5 % Triton X-100 three times.

For western blotting, samples were run on 7.5% SDS-PAGE gel and electrotransferred to a nitrocellulose membrane (HiBond ECL, Amersham).

x) Immunolocalization

Immunolocalization studies in worm embryos were performed as previously described ref. In brief, gravid hermaphrodites were placed on aminopropyl-silane treated slides, a coverslip was added, and sufficient pressure to extrude the embryos was applied (Jantsch-Plunger et al., 2000). The slide was placed into liquid nitrogen. The coverslip was removed while the sample was still frozen, the preparation was fixed with -20°C methanol, and antibody staining was performed according to standard procedures. Immunolocalization studies using HeLa cells were performed according to standard procedures following fixation in -20 °C methanol.

15 Example 6

Central spindle assembly is independent of RhoA

The process of central spindle assembly is dependent on the RhoGAP protein CYK-4. In the previous Examples, it was found that RhoA is essential for cytokinesis while Rac and Cdc42 are not (Jantsch-Plunger et al., 2000). Since RhoA is required for cytokinesis, we tested if this GTPases is also required for central spindle assembly. To inactivate RhoA we grew worms on bacteria expressing dsRNA from the coding region of the gene (Timmons and Fire, 1998). Embryos were fixed and immunofluorescence-stained for CYK-4, microtubules and DNA with anti-CYK-4 antibody, anti- α -tubulin antibody and Hoechst 33342). In both control embryos and

rhoA(RNAi) embryos, central spindles formed and were prominently labeled with anti-CYK-4 antibodies.

Although RhoA dsRNAi causes a fully penetrant inhibition of cytokinesis, central spindle assembly
5 occurs normally, and CYK-4 localizes to the central spindle (figure 11). Thus the requirement for RhoA in cytokinesis can not be accounted for by a requirement for RhoA in central spindle assembly. These data
10 indicate that the requirement for CYK-4 in central spindle assembly is independent of its ability to regulate RhoA.

Example 7

CYK-4 and ZEN-4 form a complex in vivo and in vitro

15 Since central spindle assembly and cytokinesis requires both CYK-4 and ZEN-4 and these two proteins are interdependent for their proper localization, it is possible that these factors exist in a stable biochemical complex. In order to test this
20 possibility, we performed immunoprecipitation experiments using extracts prepared from early *C. elegans* embryos. When embryo extracts were immunoprecipitated using anti-CYK-4 antibodies, we found that significant amount of ZEN-4 co-
25 immunoprecipitated suggesting the existence, in vivo, of a stable complex containing CYK-4 and ZEN-4 (fig. 12A).

To determine whether CYK-4 and ZEN-4 are competent to interact in the absence of other nematode proteins, the

two proteins were produced by in vitro translation and subjected to immunoprecipitation. When CYK-4 and ZEN-4 (fused to the chitin binding domain (CBD)) were translated in vitro and subsequently mixed, both proteins could be recovered in high yield by chitin beads (fig. 12B). The co-precipitation of CYK-4 was dependent on ZEN-4; CYK-4 was not recovered on chitin beads when ZEN-4 was substituted by CBD alone. Moreover, when luciferase was substituted for CYK-4, it did not co-precipitate with ZEN-4. The association between CYK-4 and ZEN-4 could also be detected when anti-CYK-4 antibodies were used to retrieve the complex (fig. 12C).

Figure 12 shows that CYK-4 and ZEN-4 bind to each other in vivo (A) and in vitro (B and C). (A) CYK-4 and ZEN-4 were immunoprecipitated from worm embryo extracts with anti-CYK-4 antibody, anti-ZEN-4 antibody, or with non specific rabbit IgG as a control. The immunoprecipitates were resolved on SDS-PAGE gels followed by western blotting with an anti-ZEN-4 antibody. Mock immunoprecipitation without extract was included to control for cross reactivity with the antibodies. ZEN-4 specifically co-immunoprecipitated with CYK-4. (B) ³⁵S-labeled CYK-4 (or luciferase, LUC) and chitin-binding domain (CBD)-tagged ZEN-4 (or CBD alone) were separately expressed by in vitro translation (left panel). Translation reactions were mixed and incubated in the indicated combinations and CBD-ZEN-4 or CBD alone were recovered with chitin beads (right panel). In this and all gels in this paper the input lanes contain the same amount of translation product as was added to the beads used for

precipitation. The precipitates were resolved on SDS-PAGE gels and the labeled products detected using a phosphoimager. CYK-4 co-purified with CBD-ZEN-4 while the luciferase control did not. (C) CYK-4 (or
 5 luciferase) and CBD-ZEN-4 (or CBD alone) were co-expressed as ³⁵S-labeled proteins in the indicated combinations (left panel). CYK-4 was immunoprecipitated with an anti-CYK-4 antibody (right panel). ZEN-4 co-immunoprecipitated with CYK-4.

10

Example 8

Delineation of the region of CYK-4 necessary to bind to ZEN-4

Since the binding between CYK-4 and ZEN-4 could be
 15 easily reconstituted using in vitro translated proteins, we used this simple assay to dissect the regions of the two proteins that mediate this interaction. We first concentrated on CYK-4. The N-terminal 30 amino acids of CYK-4 are poorly conserved
 20 and are followed by a 90 amino acid region predicted to form a coiled-coil (see schematic fig. 13D). The C-terminal 250 amino acids contains a RhoGAP domain that is active in vitro against RhoA, Rac, and Cdc42 (Jantsch-Plunger et al., 2000). The RhoGAP domain is
 25 preceded by a C1 domain, a cysteine-rich domain that mediates interactions with diacylglycerol (Hurley and Meyer, 2001) other ref. Truncations of the C-terminus of CYK-4 revealed that the ZEN-4 binding region is
 30 (fig. 13A). Further truncations within this region

indicated that residues 1-120 (from the N-terminus to the end of the coiled-coil region) are sufficient to bind efficiently to ZEN-4 (fig. 13B). Deletion of the N-terminal 35 amino acids of CYK-4 prevented the
5 interaction with ZEN-4. A near full length version of CYK-4 lacking only the N-terminal 35 amino acids does not bind ZEN-4 (fig 13C). Thus the ability of CYK-4 to interact with ZEN-4 depends on the N-terminal 120 residues of CYK-4 (fig. 13D).

10 Figure 13 shows the results of the in vitro binding assay that was used to define the ZEN-4 binding region of CYK-4.

(A) Full-length ZEN-4 and C-terminal deletion fragments of CYK-4 (1-232, 1-373 and 1-468) were separately
15 expressed as ³⁵S-labeled proteins by in vitro translation (left two panels). The reaction products were mixed and after incubation ZEN-4 was immunoprecipitated with an anti-ZEN-4 antibody (right panel). The three fragments of CYK-4 co-
20 immunoprecipitated with ZEN-4. (B) Full length CYK-4 (1-681) and CYK-4 with N-terminal deletion (35-681) were expressed as ³⁵S-labeled proteins and mixed with unlabeled CBD-ZEN-4 (left panel). After incubation, CBD-ZEN-4 was affinity purified on chitin beads (right
25 panel). CYK-4 (35-681) did not copurify with CBD-ZEN-4, but full length CYK-4 did. (C) CBD-CYK-4 fragments (1-232, 35-232, 121-232, 1-120 and 35-120) and full length ZEN-4 (or luciferase as a control) were co-expressed as ³⁵S-labeled proteins by in vitro
30 translation (left panel). CBD-CYK-4 fragments were affinity purified (right panel). ZEN-4 copurified with

CYK-4 (1-232) and (1-120), but not with CYK-4 (35-232), (121-232) or (35-120). (D) A schematic summary of the CYK-4 derivatives tested. The ZEN-4 binding region was defined as residues 1-120 of CYK-4. This region
5 contains a coiled coil region and a N-terminal extension.

Example 9

Delineation of the ZEN-4 region required to bind CYK-4

10 We next used the in vitro assay to define the region of ZEN-4 that binds to the N-terminal binding of CYK-4. Initially we compared the CYK-4 binding activity of full length ZEN-4 to that of three C-terminal deletion fragments of ZEN-4: the N-terminal catalytic core alone
15 (1-434), the catalytic core domain and the linker region (1-507), and a longer derivative that also includes the coiled coil domain (1-603). The full length protein bound CYK-4 with high affinity, as did the next longer fragment (1-603), but the two smaller
20 fragments did not interact with CYK-4 (fig. 14A). Next we examined whether the binding activity could be further localized within the central region of ZEN-4. A minimal binding domain consisting of 169 amino acids was defined (fig. 14B). This region appears to consist
25 of two elements, the linker region and the coiled coil; neither of these individual elements had detectable binding activity on their own.

The interaction of CYK-4 with the neck linker/coiled coil region of ZEN-4 is particularly interesting in

light of recent studies that indicate that in conventional kinesin, the neck linker region is critical for transducing chemical energy into mechanical energy (Case et al., 2000; Rice et al.,
5 1999) (see discussion).

Figure 14 shows the results of the in vitro binding assay that was used to show that the central region of ZEN-4 is necessary and sufficient to bind to CYK-4.

Full length ZEN-4 (1-775) and C-terminal deletion
10 fragments of ZEN-4 (1-603, 1-507 and 1-434) were ³⁵S-labeled by in vitro translation and incubated with unlabeled CYK-4 (or luciferase as a control) (left panel). CYK-4 was precipitated with anti-CYK-4 antibody (right panel). Full length ZEN-4 and ZEN-4
15 (1-603) co-precipitated with CYK-4, while shorter fragments (1-507, 1-434) did not. (B) CBD-tagged fragments of ZEN-4 (1-603, 1-434, 435-603, 435-507 and 503-603) were expressed in an unlabeled form and incubated with ³⁵S-labeled CYK-4 (1-232) (left panel).
20 ZEN-4 fragments were pulled down by affinity chromatography using chitin beads (right panel). CYK-4 (1-232) copurified with ZEN-4 (1-603) and (435-603), but not with ZEN-4 (1-434), (435-507) or (503-603).
(C) A schematic summary of the ZEN-4 derivatives
25 tested. ZEN-4 fragments containing residues (435-603) associate with CYK-4, while the fragments lacking this region did not. This region contains a "neck" region C-terminal to the kinesin catalytic core and a coiled coil region.

Example 10

a) The product of *cyk-4(t1689ts)* does not interact with ZEN-4.

Although the amino terminal region of CYK-4 exhibits
5 poor sequence conservation at the primary sequence
level, our earlier studies had indicated that this
region is crucial for function since the mutant allele
that was used to identify the *cyk-4* gene had a Ser to
Leu substitution at position 15. Since this point
10 mutation maps into the ZEN-4 binding determinant and
since, in vivo, this mutation seems to affect the
ability of CYK-4 and ZEN-4 to colocalize, we tested
whether this mutation also affects the ability of these
two proteins to interact in vitro. The S15L mutation
15 was introduced into the amino terminal fragment of
CYK-4 and used for ZEN-4 binding assays. Whereas an
interaction between the N-terminal domain of CYK-4 and
the central region of ZEN-4 can be readily detected,
the S15L mutant does not interact with ZEN-4
20 (fig. 15B). This experiment indicates that the primary
cause for the defect in *cyk-4(t1689ts)* is a defect in
the interaction with ZEN-4.

b) In vivo, ZEN-4 is in a stoichiometric complex with
Cyk-4

25 To gain further insight into the molecular architecture
of the CYK-4/ZEN-4 complex and to determine whether a
the majority of ZEN-4 is in a complex with CYK-4 in
vivo, we performed sucrose density gradient
centrifugation experiments with extracts prepared from
30 *C. elegans* embryos. When wild-type extracts are

prepared in the presence of 0.6 M NaCl, ZEN-4 migrates as a symmetrical 9 S peak (fig. 15C). CYK-4 migrates at an identical S-value (data not shown). To determine whether the migration of ZEN-4 on sucrose gradients was dependent on its association with CYK-4, we took advantage of the finding that the *cyk-4(t1689)* allele is specifically defective in its ability to bind to ZEN-4. When extracts were prepared from *cyk-4* mutant embryos and subjected to sucrose density centrifugation, ZEN-4 migrates as a symmetrical peak with a sedimentation value of 6 S, significantly less dense than ZEN-4 sediments in wild type extracts (fig. 15C). These data indicate that, in vivo, the majority of ZEN-4 is in a complex with CYK-4.

c) In vivo evidence for the CYK-4/ZEN-4 interaction domains

To gain further insight into the mechanism of CYK-4 function in vivo, we sought to identify extragenic suppressors of the *cyk-4(t1689ts)* mutation. Homozygous *cyk-4(t1689ts)* animals were mutagenized and allowed to self fertilize for two generations at the permissive temperature. When the F2 animals reached early adulthood, the temperature was shifted to 20°C to impose a selection for fertile animals carrying presumptive suppressor mutations. By screening through approximately 100,000 F1 genomes, we identified 18 suppressor mutations (see methods). Intragenic suppressors were identified by sequencing of the *cyk-4* locus PCR amplified from suppressor strains. Six suppressor strains contain additional point mutations in the N-terminus of CYK-4; all of the mutations code

In addition to the mutations found within the *cyk-4* gene itself, we also identified extragenic suppressor mutations. One suppressor strain that does not contain any mutations in the *cyk-4* gene was characterized in more detail. The suppressor activity was mapped using single nucleotide polymorphisms (SNPs) to the central region of chromosome IV (see methods). Since the *zen-4* gene maps to this region, the coding region of the *zen 4* gene was sequenced. This strain, *xs82*, was found to contain a point mutation that causes a substitution of glutamic acid for a lysine at position 502 (fig. 15A). Importantly, this substitution maps directly to the region of *ZEN-4* that was found to mediate the interaction with *CYK-4* in vitro. To confirm that the substitution in *zen-4(xs82)* is responsible for the suppressing activity, we mapped the position of the suppressor to less than 1 cM surrounding the *zen-4* locus. This mapping data combined with the fact that the sequence polymorphism maps to the *CYK-4* binding region suggests strongly that these point mutations are responsible for the suppressing activity.

To determine whether the substitution, E502K, has any phenotype on its own, in the absence of the *cyk-4(t1689)* allele, we crossed out *cyk-4(t1689)* and identified worms homozygous for *zen-4(xs82)*. These worms grew normally at 16°C, 20°C, and 25°C.

d) ZEN-4(xs82) partially restores binding to CYK-4 in vitro

As mentioned above, CYK-4 S15L is unable to interact with ZEN-4 in the in vitro binding assay. If the
5 immediate consequence of the S15L substitution in the N-terminus of CYK-4 is defective ZEN-4 binding, then an extragenic suppressor mutation in *zen-4* that rescues *cyk-4(t1689)* might be expected to restore binding to CYK-4 S15L. To test this possibility we translated a
10 N terminal fragment of CYK-4 carrying the S15L point mutation and mixed this product with a C terminal fragment of ZEN-4 carrying either the wild-type glutamic acid at position 502 or the lysine substitute allele. The wild-type CYK-4 fragment bound equally well
15 to wild-type and the E502K derivative of ZEN-4. As discussed above the CYK-4 S15L is unable to bind to wild-type ZEN-4. However, CYK-4 S15L was able to bind to ZEN-4 E502K, though this interaction was weaker than that seen with the wild-type proteins (fig. 15A). These
20 data, together with the genetic suppression, prove that the interaction between the N-terminus of CYK-4 and the central region of ZEN-4 is crucial for its function in vivo.

Figure 15 shows that a complex of CYK-4 and ZEN-4 is
25 required for function in vivo

(A) A screen for suppressors of *cyk-4(t1689ts)* identified a point mutation of *zen-4(xs82)*. This allele has a G to A point mutation, resulting in substitution of glutamate at residue 502 to lysine.

30 (B) The E502K mutation partially restores binding to

CYK-4 S15L. CYK-4 fragments (1-232, 1-232 with a point mutation S15L, 35-232) were expressed as ^{35}S -labeled protein and incubated with unlabeled ZEN-4 fragments (435-775, 435-775 with the E502K mutation, 609-775).
5 ZEN-4 fragments were precipitated with anti-ZEN-4 antibody. CYK-4 (1-232) co-precipitated with ZEN-4 (435-775) but not with ZEN-4 (609-775) as shown in Figure 13. The S15L mutation in CYK-4 abolishes its ability to bind to ZEN-4 (CYK-4 1-232 S15L and ZEN-4
10 435-775 wt). However, the suppressor mutation in ZEN-4 (E502K) partially recovered this interaction ((CYK-4 1-232 S15L and ZEN-4 435-775 E502K). The ZEN-4 E502K mutation does not affect the binding to wild-type CYK-4. (C) The majority of ZEN-4 is in a complex with
15 CYK-4 in vivo. Extracts were made from wild type (N2) embryos or *cyk-4(t1689ts)* embryos. Sedimentation of ZEN-4 was analyzed by sucrose density gradient centrifugation followed by western blotting. The samples were spiked with the indicated standards and
20 they ran identically in the two gradients. ZEN-4 from wild type embryos sediments at 8 S, whereas ZEN-4 from *cyk-4* mutant embryos sediments at 7S.

Example 11

25 CYK-4 and ZEN-4 self-associate

Thus far we have established that central spindle assembly is dependent on an interaction between CYK-4 and ZEN-4 and have defined the regions of both proteins that are required for this interaction. Interestingly,
30 both binding domains contain coiled coil regions; these

regions are required for binding activity. Since coiled coils are often dimerization motifs, and since kinesin motors are often composed of two motor domains tethered together through a coiled coil, we examined
5 whether CYK-4 and ZEN-4 are individually able self-associate.

To assess if CYK-4 self associates, we co-expressed full length CYK-4 and the CYK-4 derivative 1-232. As a control, the full length protein was substituted by a
10 truncated version lacking the N-terminal 120 amino acids. The longer CYK-4 fragments were immunoprecipitated with an antibody directed against the C-terminus of CYK-4 and the co-immunoprecipitation of the short N-terminal fragment was assessed. The
15 N-terminal fragment bound to full length CYK-4 but not to CYK-4 lacking the N-terminal domain (figure 16A). These data demonstrate that CYK-4 self associates. This association is likely mediated by the coiled-coil domain.

20 The capacity of ZEN-4 to self-associate was examined using a similar strategy. Full length ZEN-4 was co-expressed with affinity-tagged deletion derivatives of ZEN-4. Neither the N-terminal region (1-507) nor the C-terminal region (604-775) of ZEN-4 bound appreciably
25 to full length ZEN-4, but two internal fragments of ZEN-4 (503-775; 435-603) did bind to full length ZEN-4 (figure 16B). The region common to these two fragments consists of residues 503-603 which is the region predicted to form a coiled coil domain.

The ability of CYK-4 and ZEN-4 to self associate in vitro raise the possibility that in vivo, the CYK-4/ZEN-4 complex may contain, at a minimum, two molecules each of CYK-4 and ZEN-4.

5 Figure 16 shows the self-association of both CYK-4 and ZEN-4

(A) CYK-4 (1-232), which does not contain the epitope recognized by the anti-CYK-4 antibody, was co-expressed as a ³⁵S-labeled protein with CYK-4 (1-681) or
 10 (121-681), which do contain the epitope for this antibody (left panel). CYK-4 (1-681) and (121-681) were immunoprecipitated. CYK-4 (1-232) co-immunoprecipitated with CYK-4 (1-681), but not with CYK-4 (121-681). (B) CBD-tagged fragments of ZEN-4
 15 (1-507, 503-775, 435-603 or 604-775) were co-expressed with untagged ZEN-4 (1-775) (left panel). CBD-tagged fragments were purified by affinity chromatography with chitin beads (right panel). Untagged ZEN-4 (1-775) copurified with CBD-ZEN-4 (503-775) and (435-603), but
 20 not with ZEN-4 (1-507) or (604-775). The low extent of labelling of 604-775 as compared to 435-603 is attributable to low cysteine and methionine content of the C-terminal fragment.

25 Example 12

The human orthologs of CYK-4 and ZEN-4, HsCYK-4 and MKLP1, interact in vivo and colocalize.

In *C. elegans*, CYK-4 and ZEN-4 associate in vivo and in vitro and this complex is important for central spindle

assembly and cytokinesis. To extend these findings we evaluated whether mammalian cells contain a stable complex containing the human orthologs of these proteins, HsCYK-4/MgcRacGAP (Hirose et al., 2001; Toure
5 et al., 1998; Woollorton et al., 1999) and MKLP1, respectively. Extracts were prepared from mitotic HeLa cells and antibodies directed against HsCYK-4 or MKLP1 were used for immunoprecipitation followed by western blotting. MKLP1 could be co-immunoprecipitated with
10 anti-HsCYK-4 antibodies and vice versa (fig. 17a). The immunoblots reveal that small amounts of HsCYK-4 and MKLP1 migrate with reduced mobility, likely due to phosphorylation.

To obtain evidence that these proteins play a similar
15 role in human cells as was shown previously for the nematode proteins, we examined the localization of HsCYK-4 and MKLP1 in cultured (HeLa) human cells. During all stages of the cell cycle the two proteins precisely co-localize (insets fig 17b). In early
20 anaphase, the two proteins localize to a discrete, central region of the central spindle. Three dimensional reconstruction of optically sectioned anaphase cells reveals that each bundle of microtubules in the central spindle is labeled at its distal end by
25 MKLP1 antibodies. During mid-to-late anaphase, the MKLP1 staining region is $0.93 \pm 0.25 \mu\text{m}$ in length ($n=56$). This region largely corresponds to the region that appears deficient in tubulin staining, but this is a known artifact caused by epitope masking (Saxton and
30 McIntosh, 1987). The extent of staining is somewhat narrower than previous ultrastructural determinations of the extent of microtubule overlap ($> 2 \mu\text{M}$)

(Mastronarde et al., 1993). Later in cleavage, both proteins localize to a discrete, central portion of the midbody. During interphase, some cells contain HsCYK-4 and MKLP1 in the nucleus, whereas others do not.

5 This is likely due to cell cycle regulated accumulation of these factors since, upon G2 arrest, HsCYK-4 and MKLP1 accumulate in the nucleus of all cells (data not shown). In addition, most cells contain brightly-staining, cortical ring-like structures. These
10 structures likely correspond to division remnants; previous time-lapse microscopy with GFP-tagged CYK-4 and ZEN-4 in nematode embryos revealed that the central spindle matures into the midbody that later develops into a persistent spot or ring in the cell cortex. Thus
15 there is a striking concordance between the biochemical properties and the subcellular localization of CYK-4/ZEN-4 in *C. elegans* embryos and HsCYK-4/MKLP1 in human cells. Since the names of the individual proteins vary from species to species, we propose to call this
20 complex "centralspindlin". It is reasonable to speculate that centralspindlin performs a similar function in these two systems.

To gain insight into the architecture of centralspindlin, we investigated the hydrodynamic
25 properties of its constituents. Lysates were prepared from mitotic Hela cells and run on sucrose density gradients. Western blotting of the gradient fractions indicates that HsCYK-4 and MKLP1 precisely co-migrate on the gradients with an S-value of 8 S (fig. 17b).
30 This sedimentation behavior is similar to that of centralspindlin isolated from *C. elegans* embryos. The two proteins also co-migrated on a gel filtration

column with an apparent molecular weight of 800 kD (data not shown). Gel filtration chromatography can not accurately estimate native molecular mass of asymmetric particles, however, gel filtration data, combined with S-value measurements, allows a more accurate estimation of the native molecular mass. When the experimental values of centralspindlin are combined in this way, the complex is estimated to have a native molecular weight of ~300 kDa.

10 To determine if equimolar amounts of HsCYK-4 and MKLP1 are present in centralspindlin and whether or not other proteins are also present, we immunopurified the complex from a mitotic MAP (microtubule-associated protein) fraction that was eluted with ATP and 500 mM NaCl. Antibodies specific for HsCYK-4 and MKLP1 were used in parallel for the immunoprecipitation. Coomassie blue staining of both immunoprecipitates revealed two major bands and one minor band (fig. 17C). Mass spectrometry analysis revealed that the slower migrating major band and the minor band are MKLP1 and the faster migrating major band is HsCYK-4. No additional bands reproducibly co precipitated with HsCYK-4 or MKLP1. Given that centralspindlin has a native molecular mass of 300 kD and that it does not appear to contain any proteins in addition to HsCYK-4 (70 kD) and MKLP1 (100 kD) and given that both components are capable of self-association, we conclude that centralspindlin is a tetramer containing two molecules each of HsCYK-4 and MKLP1.

Figure 17 shows that there exists a complex of MKLP-1 and HsCYK-4 in HeLa cells

HsCYK-4/MgcRacGAP and MKLP-1, the human orthologs of CYK-4 and ZEN-4, respectively, exist as a complex in mitotic HeLa cells. (A) HsCYK-4 and MKLP-1 were immunoprecipitated from HeLa cell lysate with specific rabbit antibodies (IP:HsCYK-4 or MKLP-1) and blotted with specific mouse antibodies (blot: HsCYK-4 or MKLP-1). HsCYK-4 co-immunoprecipitated with MKLP-1 and vice versa. (B) HsCYK-4 and MKLP1 co-localize to the central spindle and the midbody. HeLa cells were fixed and immunostained with anti-CYK-4, anti-MKLP1, anti-tubulin antibodies and DNA was stained with Hoechst. Shown are cells in interphase (a) and various stages of cytokinesis (b-f). In main panels, staining of CYK-4 (green), microtubules (red) and DNA (blue) was shown. In inset, staining of CYK-4 (green) and MKLP1 (red) was shown. (C) HeLa cell lysates were analyzed by sucrose density gradient centrifugation followed by blotting with anti-MKLP-1 antibody or anti-HsCYK-4 antibody. HsCYK-4 and MKLP-1 co-sediment at 8S. The lowest band reacting with the CYK-4 antibody is caused by proteolysis of the N-terminus. (D) MKLP-1 and HsCYK-4 complex was immunoprecipitated from a microtubule-binding fraction (MTfr.) of mitotic HeLa cells with specific antibodies. Major 110 kDa and 75 kDa bands were identified by mass spectrometry as MKLP-1 and HsCYK-4, respectively. The minor band at 115 kDa also corresponds to MKLP1. Broad bands around 50 kDa and bands near gel bottom are heavy (IgGH) and light (IgGL) chains of immunoglobulin leaked from antibody beads.

Table 1

genotype	stage at time of shift to 25°C	percent viable	phenotype of surviving progeny	N
N2 (wild type)	embryos	98.8%	wild-type	485
<i>cyk-4</i>	(not shifted)	70.2%	fertile	198
<i>cyk-4</i>	embryos	1.5%	sterile, highly Unc	324
<i>cyk-4</i>	L1 larvae	100.0%	sterile, highly Unc	89
<i>cyk-4</i>	L2/L3	100.0%	sterile, highly Unc	55
<i>cyk-4</i>	L4	100.0%	fertile, lay dead embryos	52
<i>cyk-4</i> , <i>xsEx1[cyk-4:GFP]</i>	embryos	59.4%	fertile	350

- 5 Except for the N2 control, the complete genotype of the strain was *unc-32(e189) cyk-4(t1689ts)*. Gravid hermaphrodites were allowed to lay embryos for 2 hours at the permissive temperature. The adult was then removed and the number of embryos counted. After 24
- 10 hours at the indicated temperature the number of unhatched embryos was then counted.

Table 2

injected locus dsRNA	percent cytokinesis defective (N)	number of time lapse recording s	number cytokinesi s defective	additional phenotypes
RhoA Y51H4A.B	95% (175)	15	15 (100%)	
Rac-1 C09G12.8 B	Not emb. lethal	5	0	
Cdc42 R07G3.1	12% (74)	22	3 (14%)	symmetric 1st division (5/22); no rotation in P1 (12/22); rotation of AB (2/22)
others F22E12.2	Not emb. lethal			
Y32F6B.3	Not emb. lethal			
K03D3.9	Not emb. lethal			

Young adult hermaphrodites were injected with the

5 indicated dsRNAs and broods of laid embryos were scored for embryonic lethality. dsRNAs that induced embryonic lethality were further characterized by dissecting embryos and scoring for multinucleate embryos and by performing time lapse recordings and evaluating

10 cytokinesis, spindle orientation in the one-cell embryo (symmetric or asymmetric first cleavage), and spindle positioning in the P1 and AB blastomeres.

Table 3

genotype	temperature	total number of stained remnants	total number of cells	average number of remnants/ cell	number of embryos
N2 (wildtype)	25°C	110	137	0.80	16
<i>zen-4(or198ts)</i>	16°C	61	83	0.73	10
<i>zen-4(or198ts)</i>	25°C (18 min.)	11	147	0.07	24
N2 (wildtype)	25°C	150	205	0.73	22
<i>cyk-4(t1689ts)</i>	16°C	61	98	0.62	10
<i>cyk-4(t1689ts)</i>	25°C (15 min.)	10	176	0.06	19

Wild-type and *zen-4(or153ts)* embryos grown at the
 5 indicated temperatures were fixed and stained for
 Cyk-4. Similarly, Wild-type and *cyk-4(t1689ts)* grown
 at the indicated temperatures were fixed and stained
 for Zen-4/CeMKlp1. The number of cells in each embryo and
 the number of remnants staining with Cyk-4 or Zen-4
 10 antibodies was counted.

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30. April 2001

Claims

1. Human polypeptide designated Cyk-4, which is a
GTPase activating protein (GAP) for Rho family of
5 GTPases, with the amino acid sequence as set forth
in SEQ ID NO:2 or with the amino acid sequence
encoded by a polynucleotide which hybridizes under
stringent conditions to a polynucleotide having a
nucleotide sequence as set forth in SEQ ID NO:1.
- 10 2. Murine Cyk-4 polypeptide designated Cyk-4, which is
a GTPase activating protein (GAP) for Rho family of
GTPases, with the amino acid sequence as set forth
in SEQ ID NO:4 or with the amino acid sequence
15 encoded by a polynucleotide which hybridizes under
stringent conditions to a polynucleotide having a
nucleotide sequence as set forth in SEQ ID NO:3.
3. An isolated DNA molecule comprising a
polynucleotide with the nucleotide sequence as set
forth in SEQ ID NO:1 encoding human Cyk-4
20 polypeptide, or an isolated DNA molecule encoding
human Cyk-4 polypeptide comprising a polynucleotide
which hybridizes under stringent conditions to a
polynucleotide having a nucleotide sequence as set
forth in SEQ ID NO:1.
- 25 4. An isolated DNA molecule comprising a
polynucleotide with the nucleotide sequence as set
forth in SEQ ID NO:3 encoding murine Cyk-4
polypeptide, or an isolated DNA molecule encoding
murine Cyk-4 polypeptide comprising a
30 polynucleotide which hybridizes under stringent

conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3.

5. An antibody which is specifically reactive with an epitope of the human Cyk-4 polypeptide of claim 1.
- 5 6. An antibody which is specifically reactive with an epitope of the murine Cyk-4 polypeptide of claim 2.
7. A method for identifying a compound capable of modulating cytokinesis, wherein the compound's ability to modulate the function of CYK-4 is
10 determined.
8. The method of claim 7 wherein the compound's ability to promote GTP hydrolysis by a Rho family GTPase is determined by incubating a substrate selected from the members of the Rho family GTPases
15 with GTP for a period of time sufficient to allow saturation of the substrate's GTP binding sites, adding Cyk-4 and allowing it to react in the presence or absence of the test compound, and determining the amount of hydrolyzed GTP.
- 20 9. The method of claim 7 wherein the compound's
} ability to inhibit Cyk-4 function is determined by determining the compound's ability to interfere with the biochemical interaction of CYK-4 and a member of the MKLP1 subfamily.
- 25 10. The method of claim 7 wherein the compound's ability to inhibit CYK-4 function is determined by determining the compound's ability to interfere with the biochemical multimerization of CYK-4.

11. The method of claim 7 wherein the compound's
ability to inhibit MKLP1 function is determined by
determining the compound's ability to interfere
with the biochemical multimerization of a member of
the MKLP1 subfamily.
- 5
12. A compound identified in the method of any one of
claims 7 to 9 for use in cancer therapy.

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Abstract

5 Mammalian CYK-4 polypeptides, which are key molecules
required for cytokinesis, and DNA molecules encoding
them. Screening methods based on the function of CYK-4
to promote GTP hydrolysis by Rho family GTPases can be
employed to identify compounds interfering with cell
10 division. Such compounds are useful in tumour therapy.

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Fig. 1

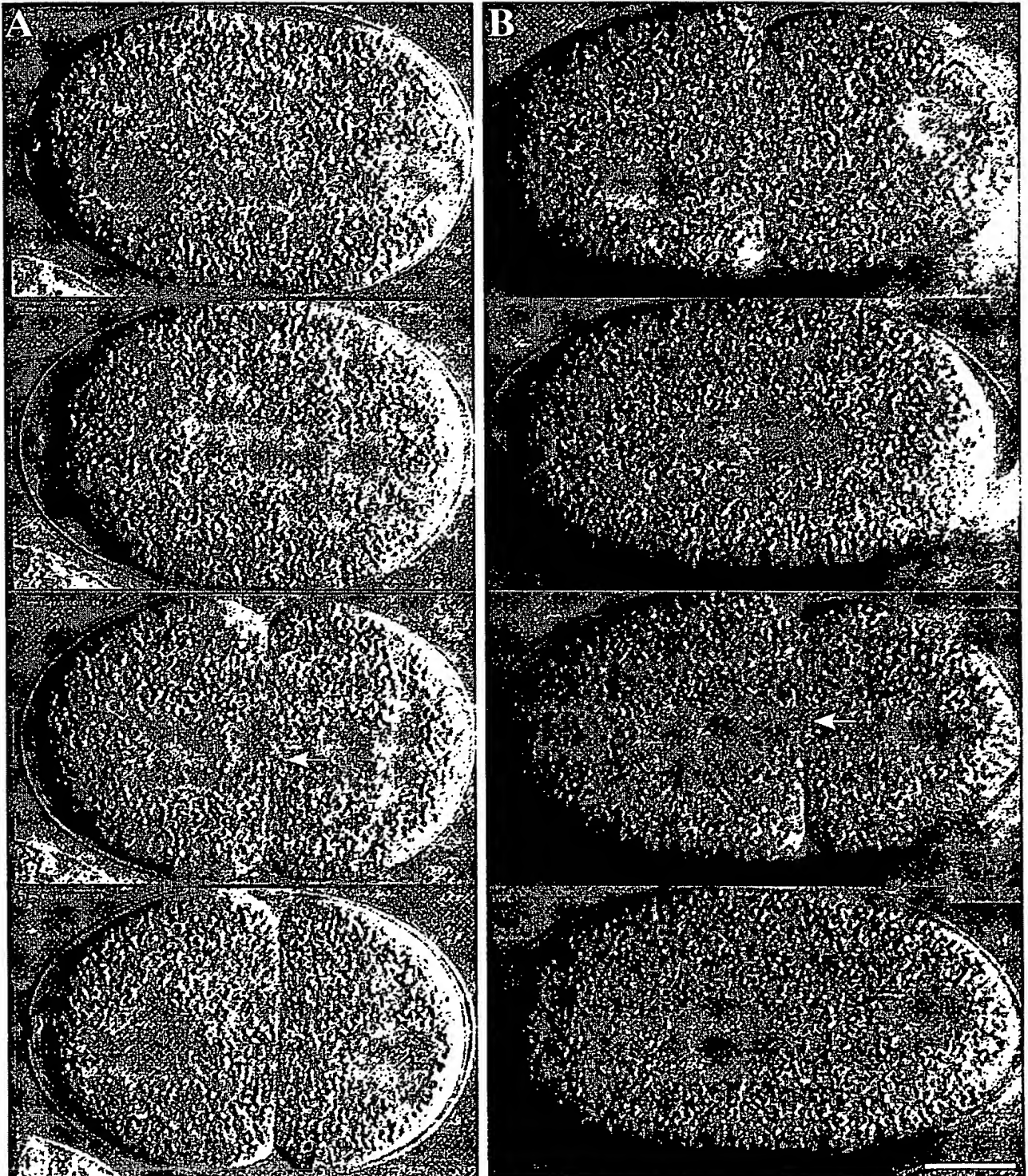


Fig. 2

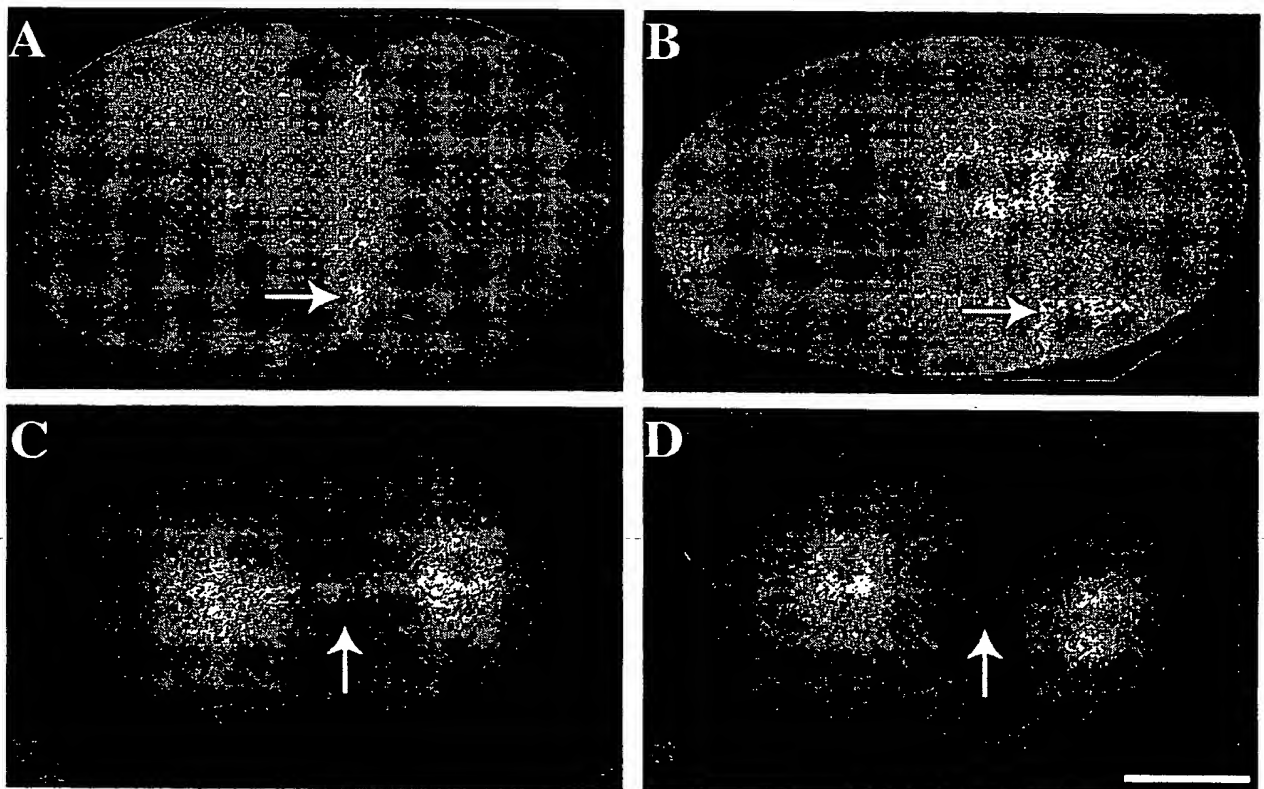


Fig. 3

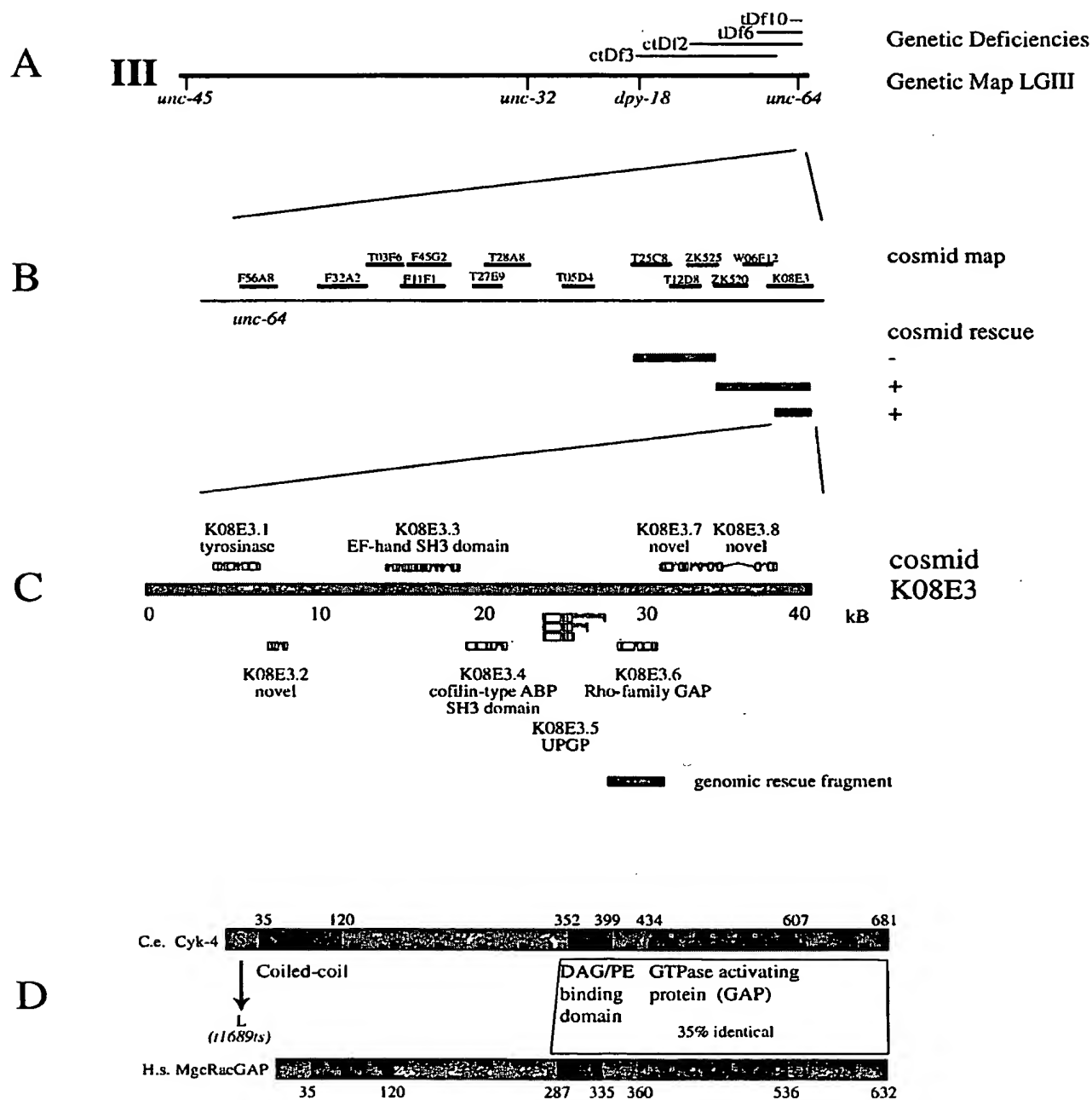


Fig. 4

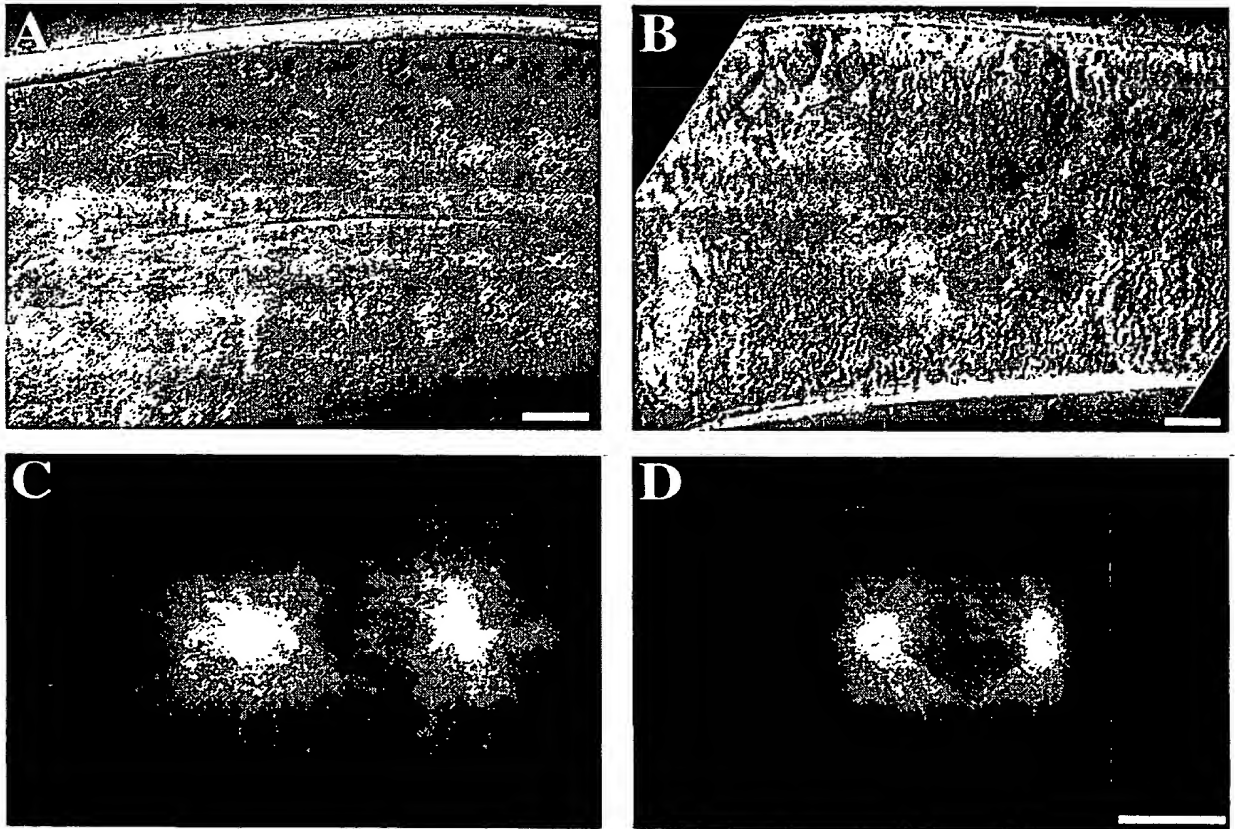


Fig. 5

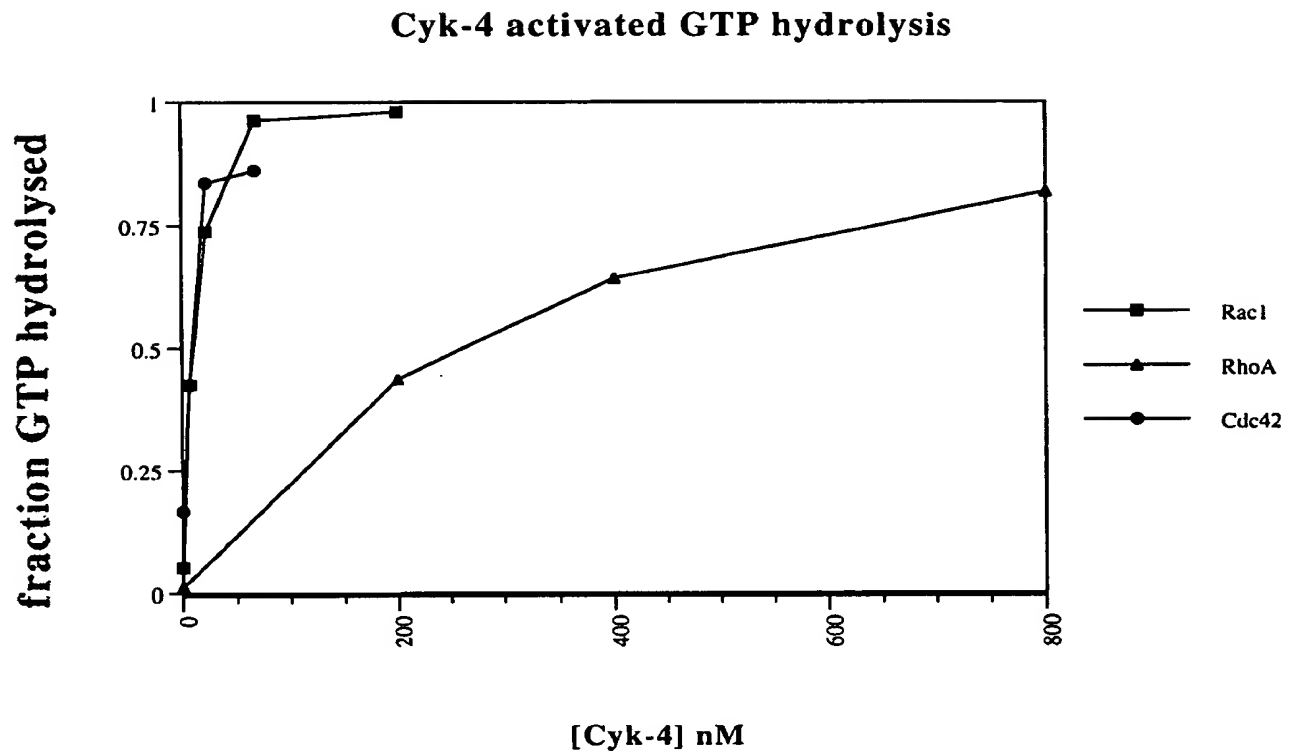


Fig. 6

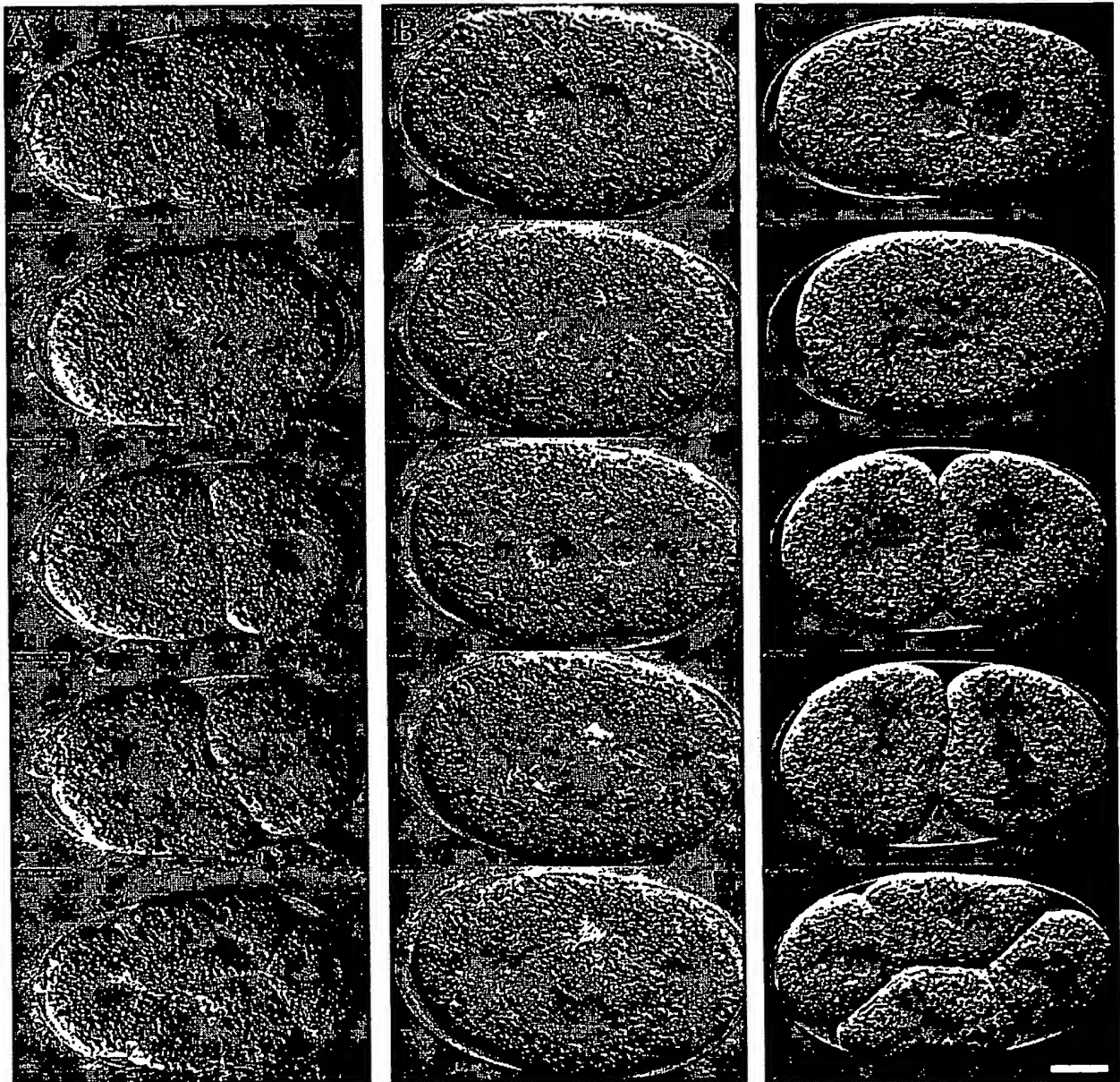


Fig. 7

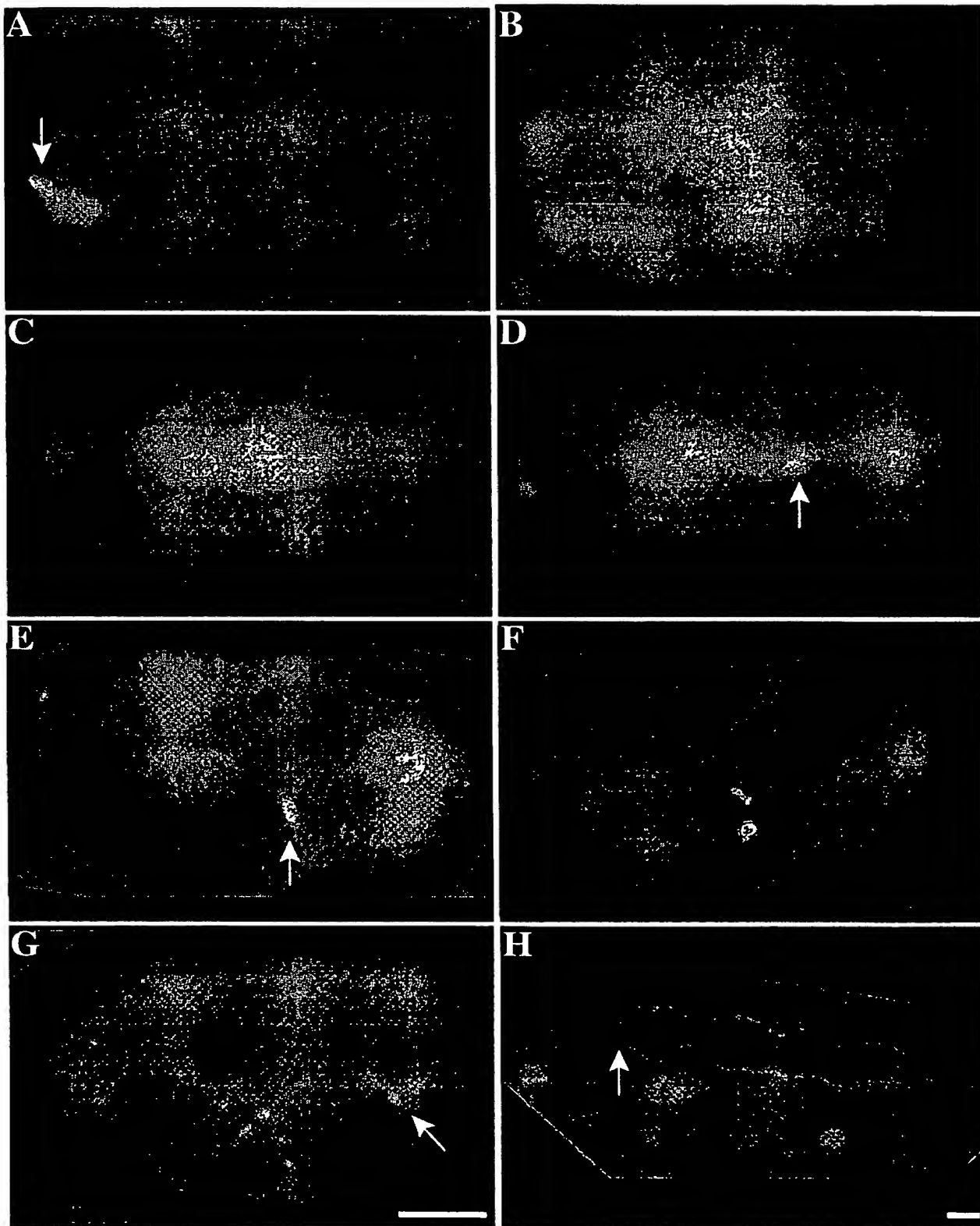


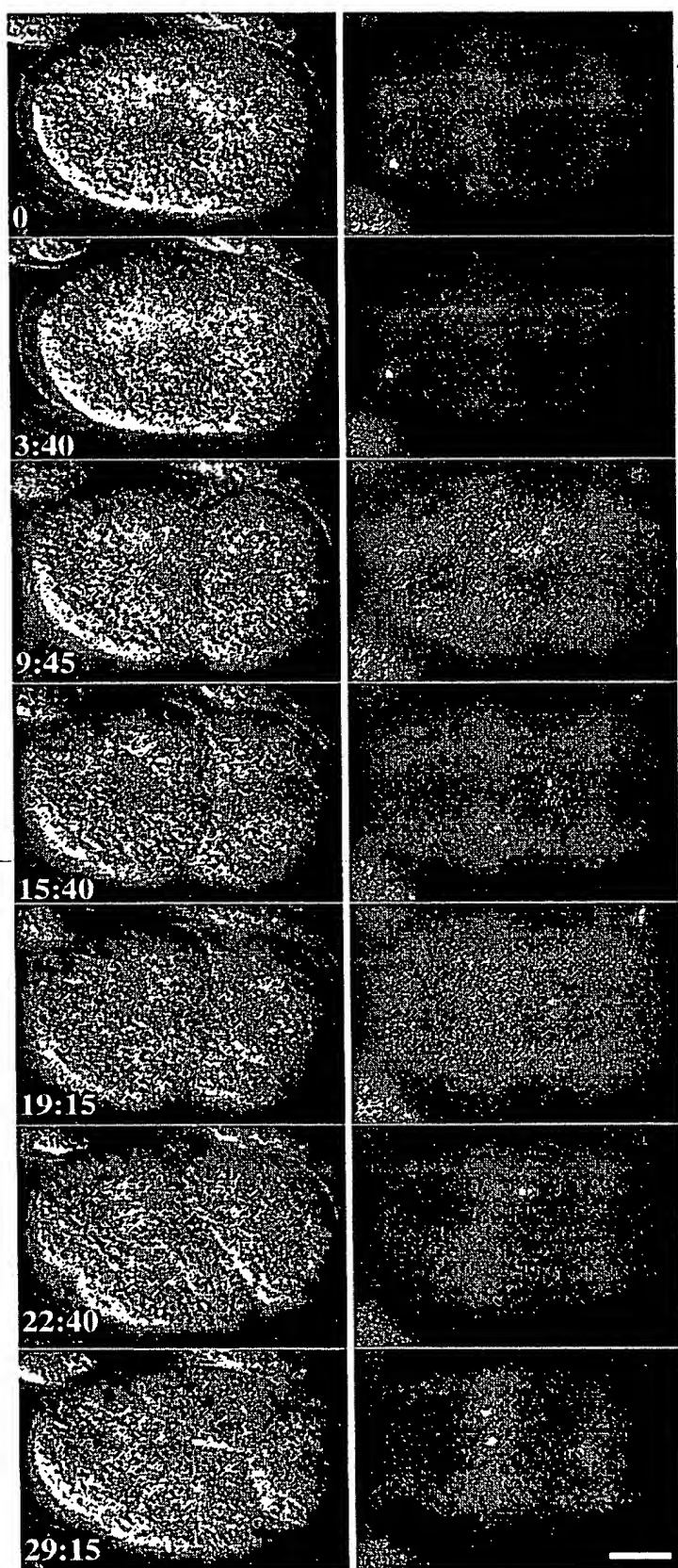
Fig. 8

Fig. 9

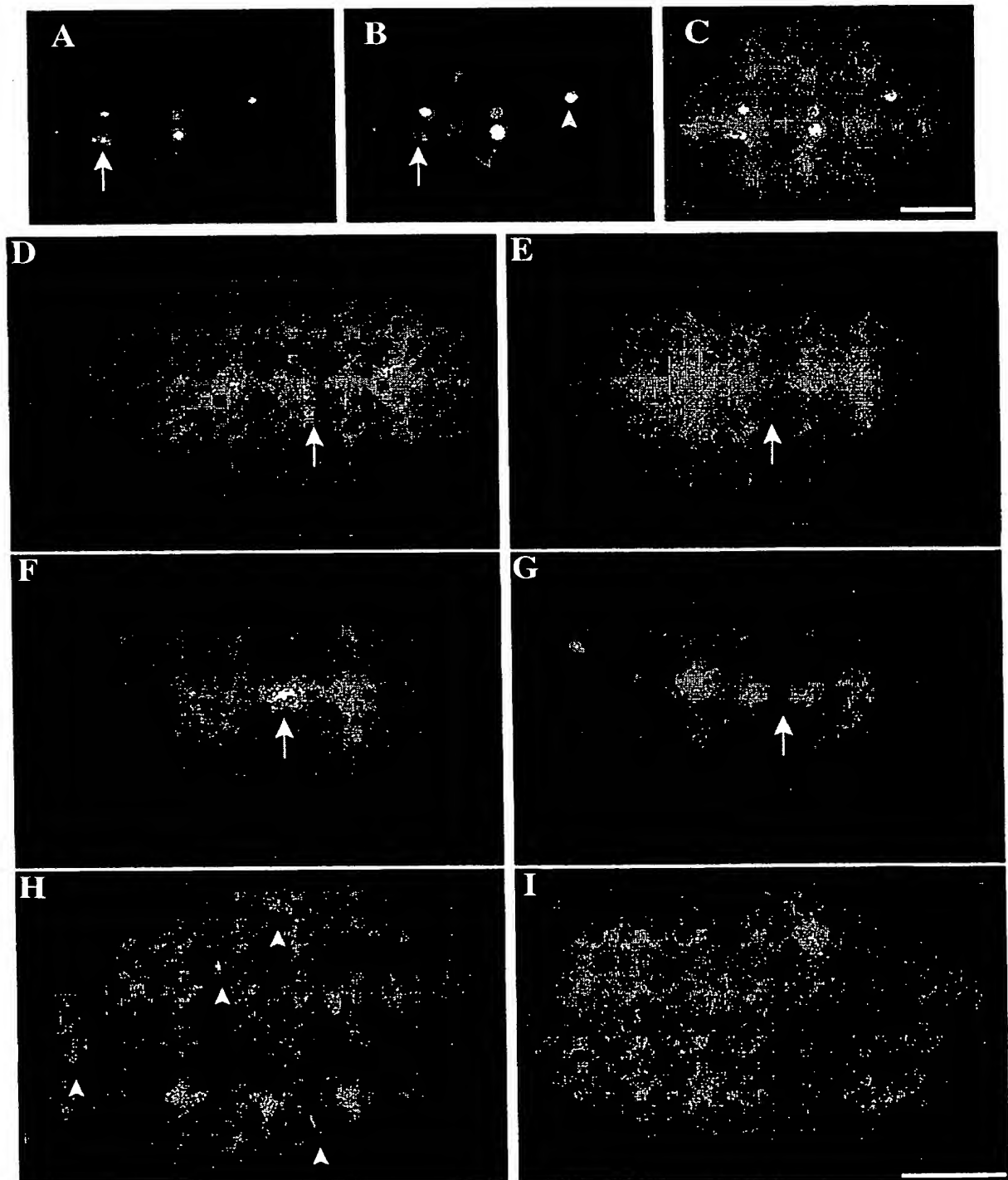


Fig. 10

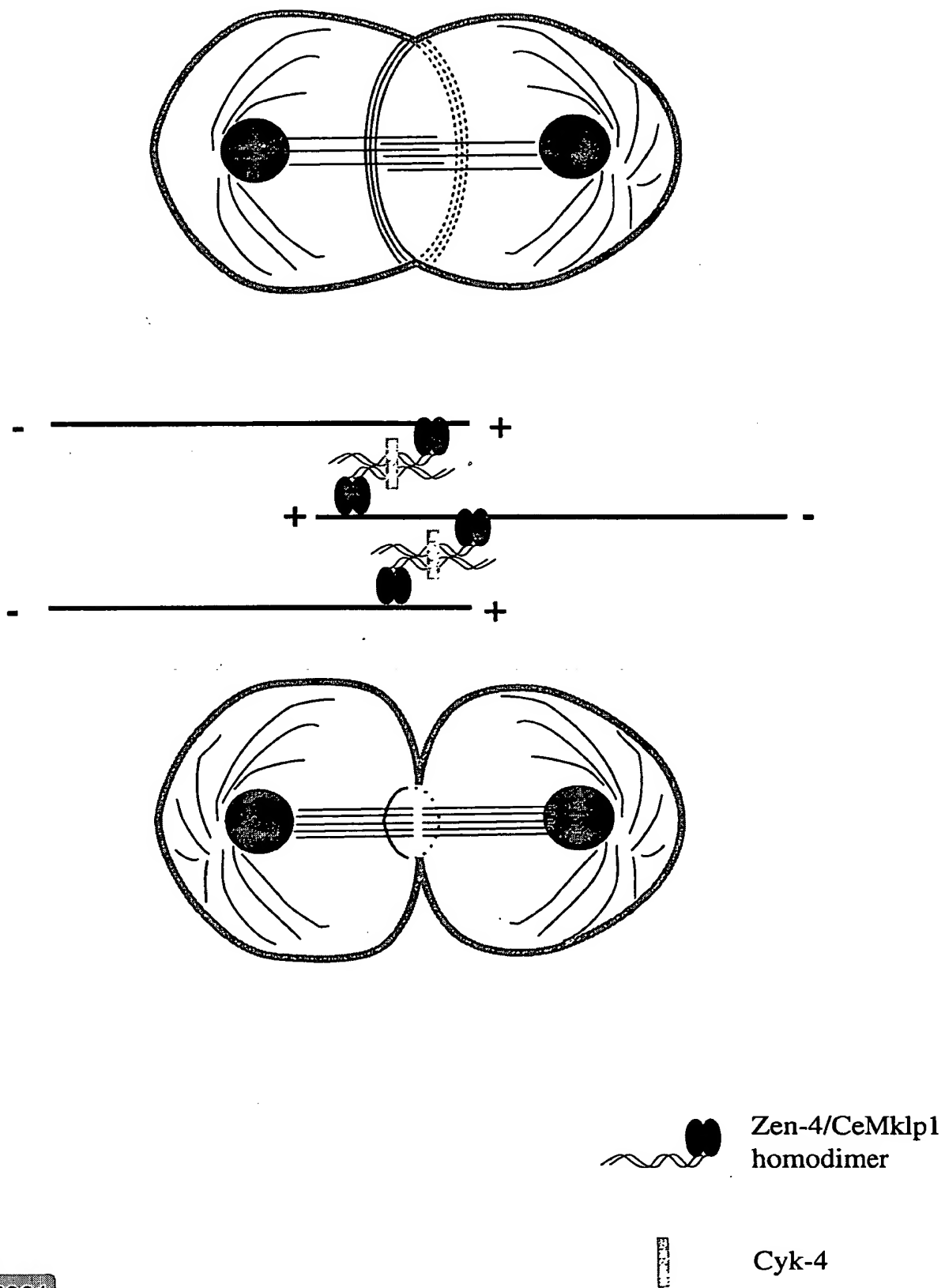


Fig. 11

rhoA (RNAi)

wt

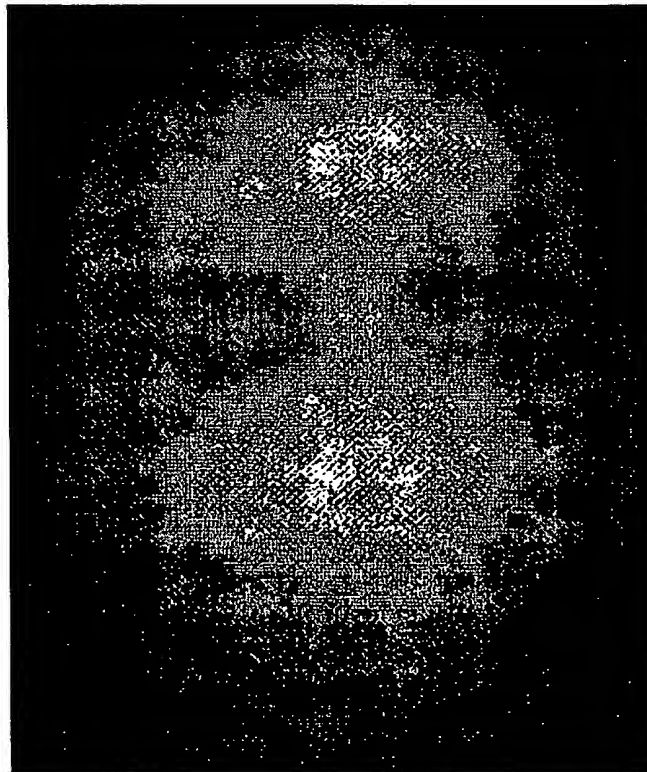


Fig. 12



Fig. 13

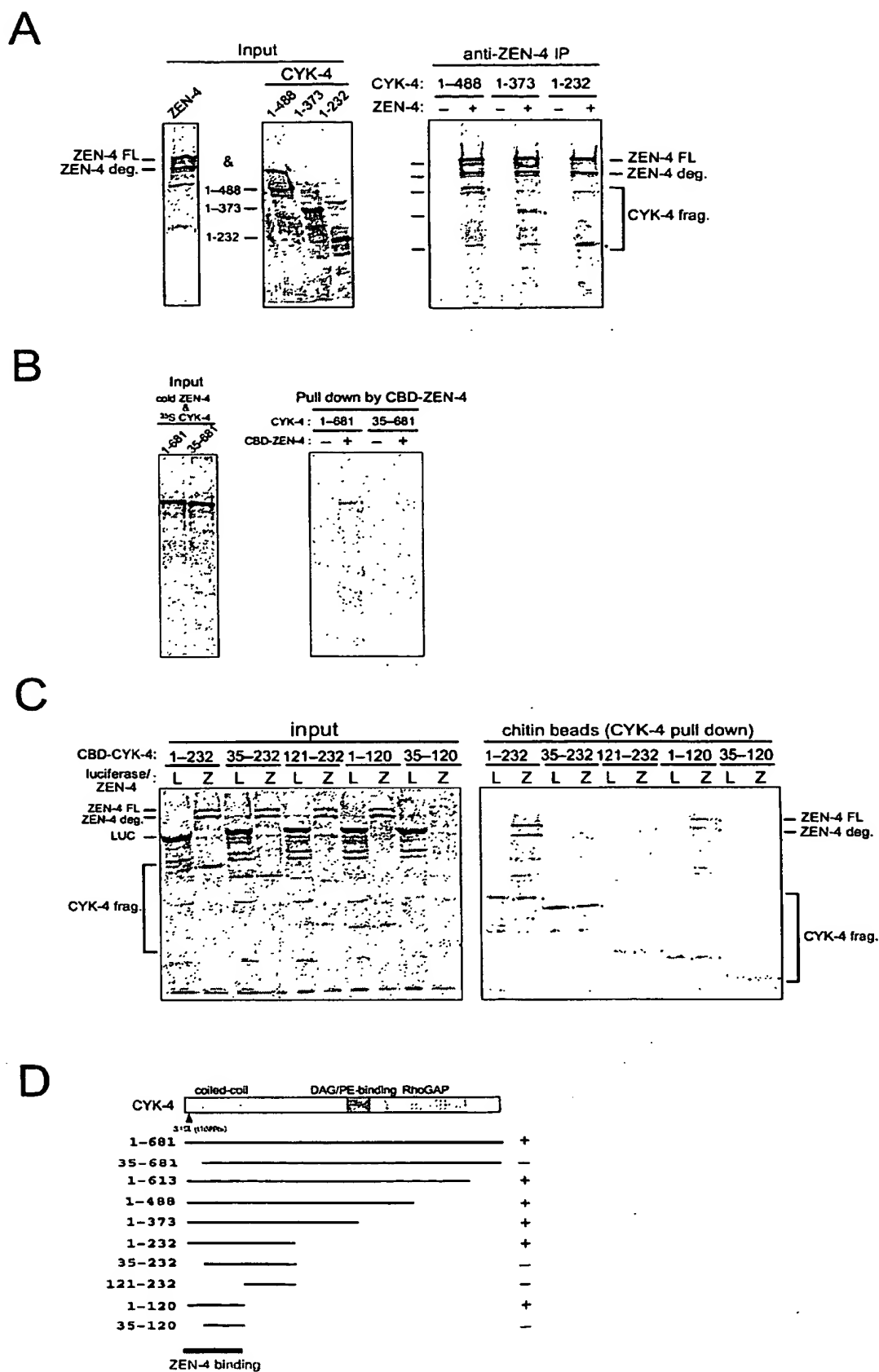
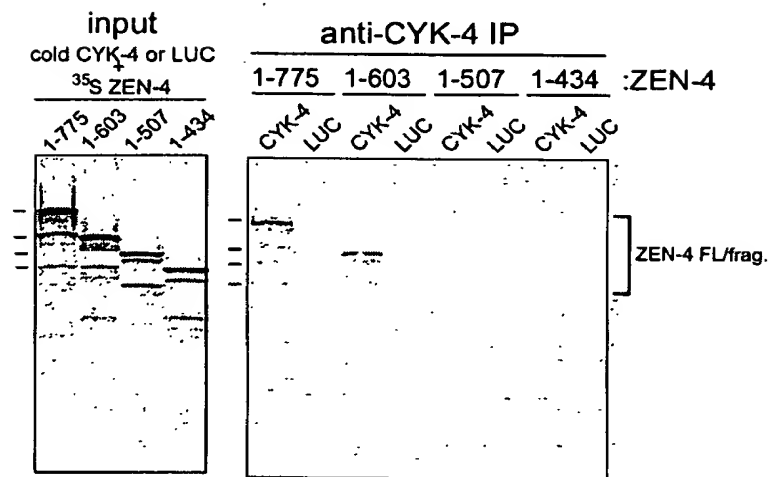
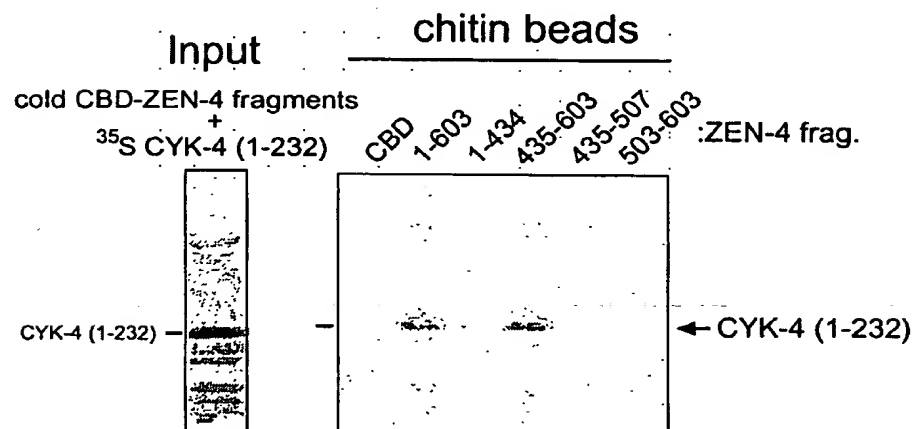


Fig. 14

A



B



C

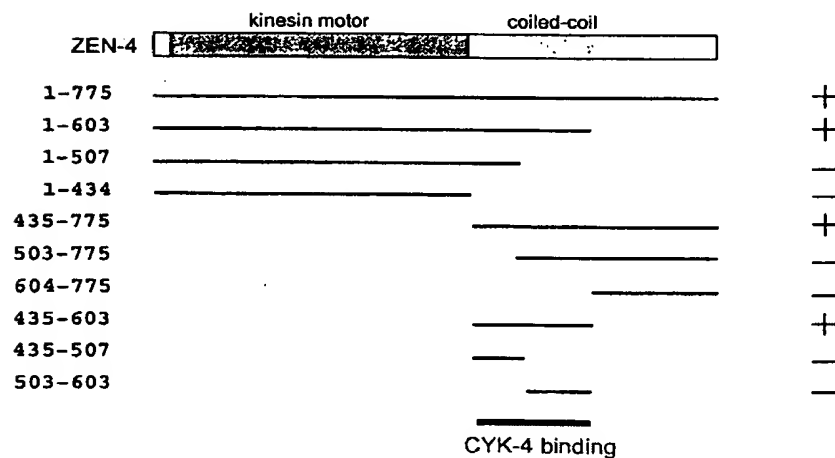
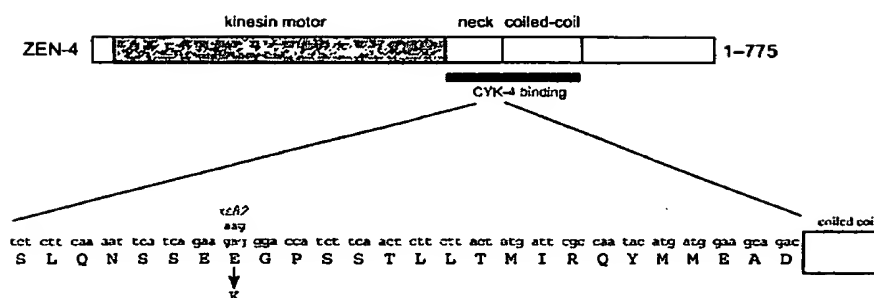
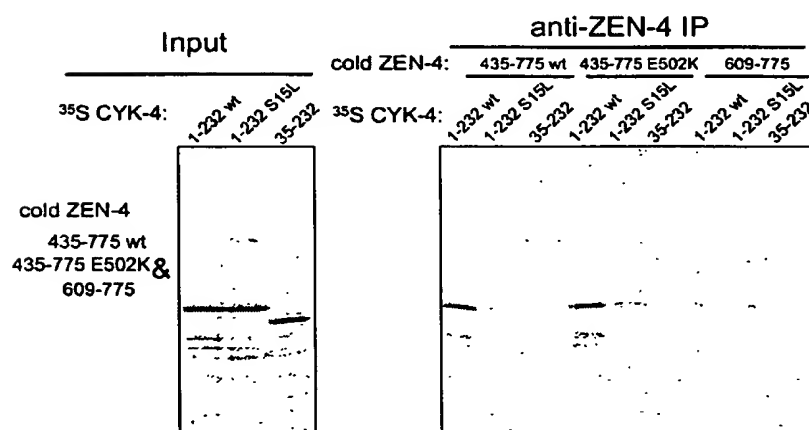


Fig. 15

A



B



C

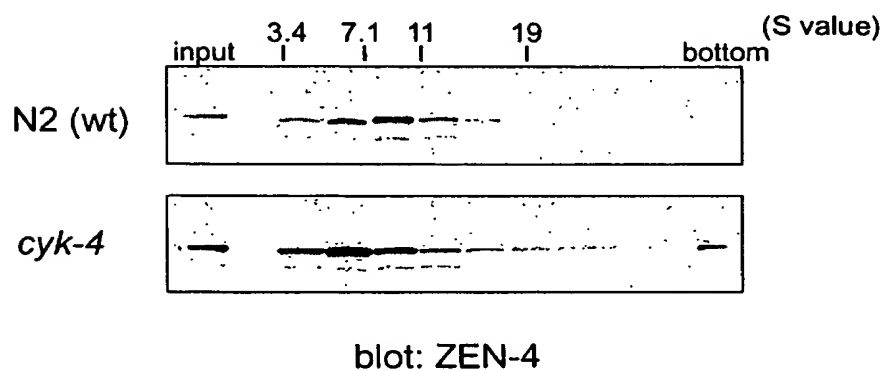
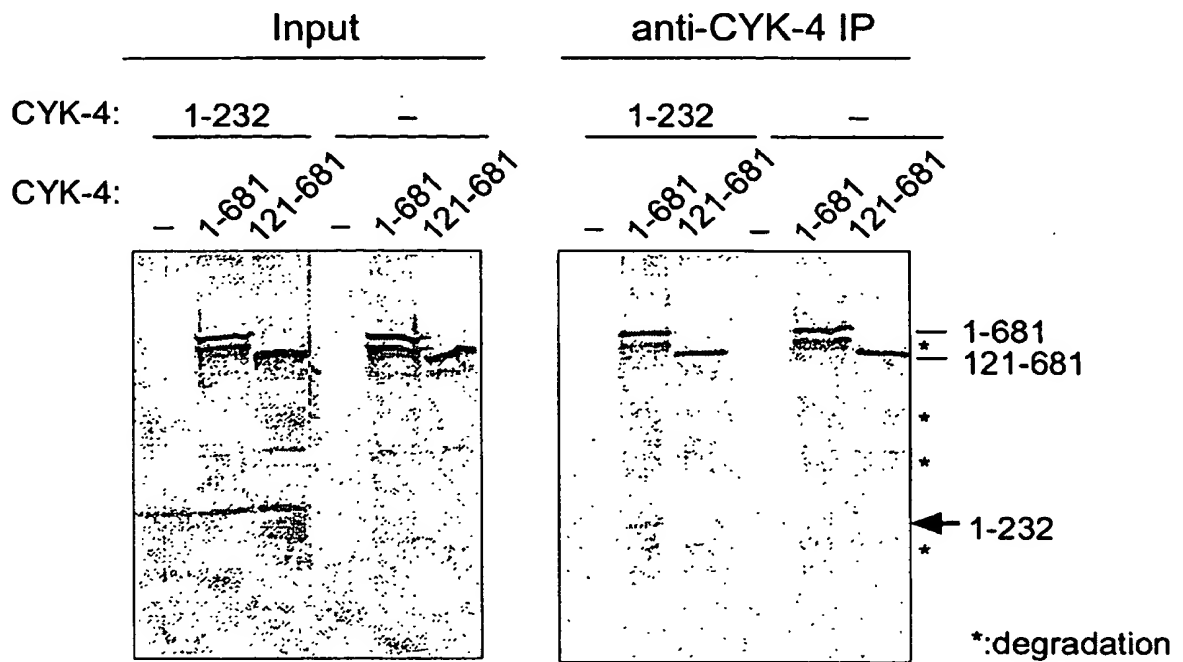


Fig. 16

A



B

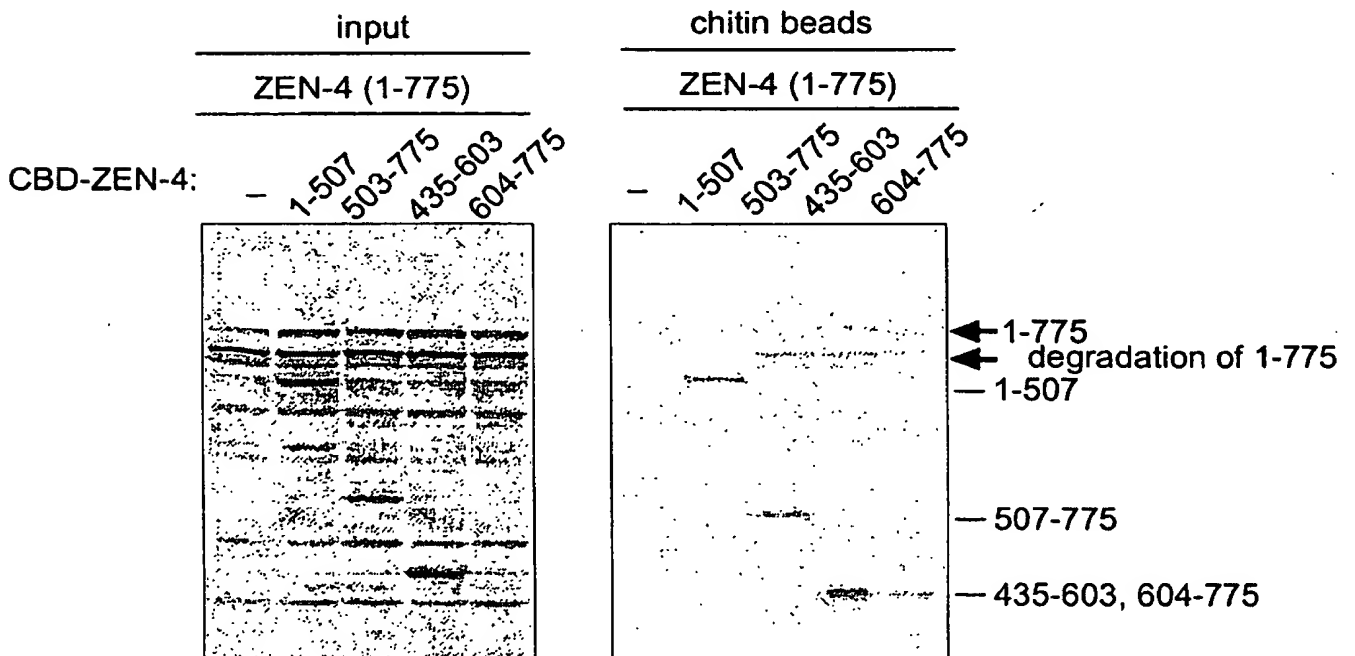


Fig. 17

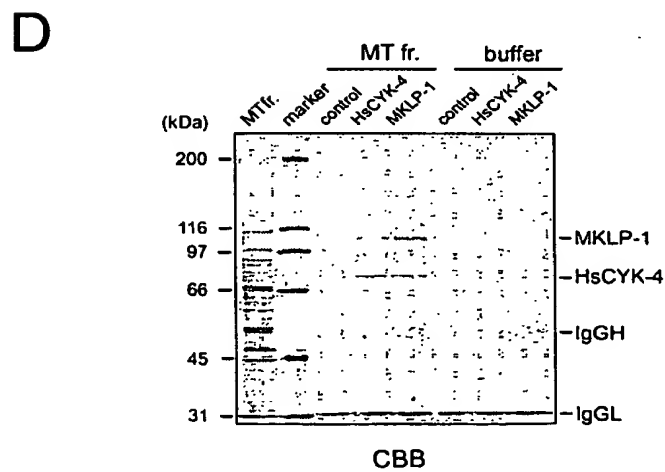
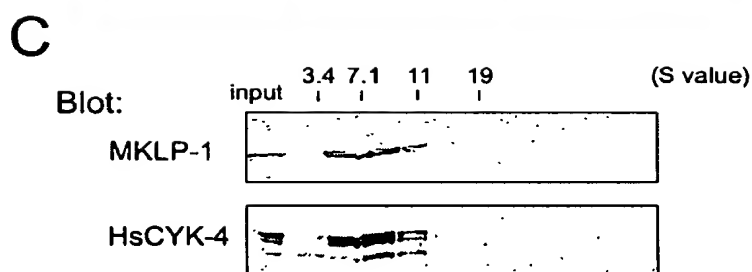
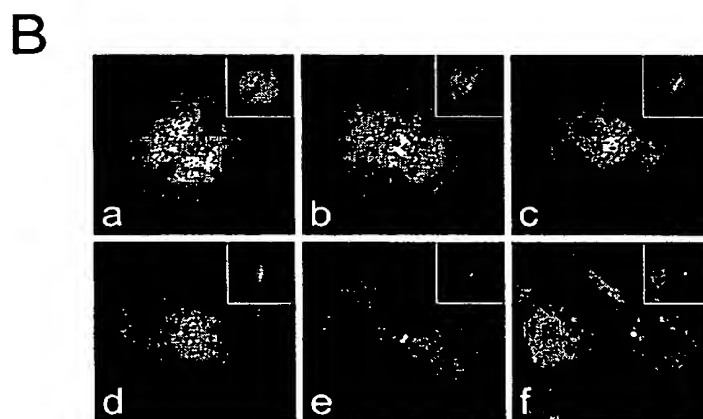
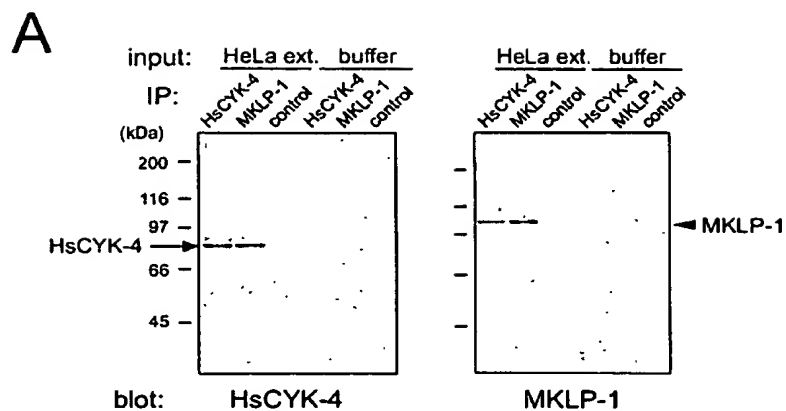
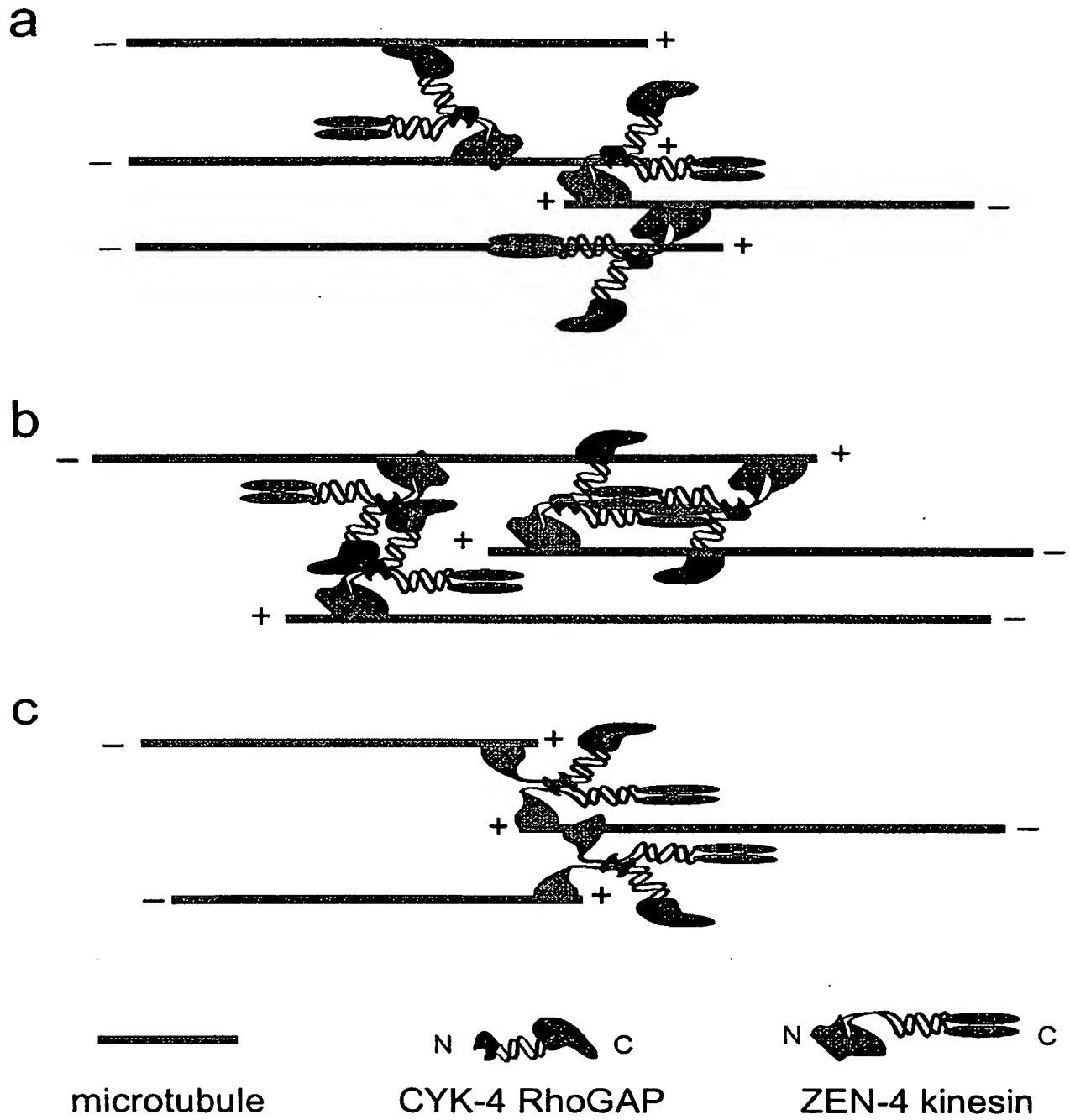


Fig. 18



EPO - Munich
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30. April 2001

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11

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Gln	Val	Val	Lys	Asp	Phe	Glu	Asp	Phe	Arg	Lys	Lys	Tyr	Gln	Arg	Thr
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Asp	Val	Glu	Ile	Lys	Arg	Arg	Gln	Arg	Ala	Glu	Ala	Glu	Cys	Ala	Lys
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Leu	Glu	Gln	Gln	Ile	Gln	Leu	Ile	Arg	Asp	Ile	Leu	Met	Cys	Asp	Thr
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12

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Thr Asp Asn Leu Gly Thr Pro Gln Asn Thr Gly Gly Met Arg Leu His
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Asp Phe Val Ser Lys Thr Val Ile Lys Pro Glu Ser Cys Val Pro Cys
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Gly Lys Arg Ile Lys Phe Gly Lys Leu Ser Leu Lys Cys Arg Asp Cys
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Arg Leu Val Ser His Pro Glu Cys Arg Asp Arg Cys Pro Leu Pro Cys
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Ile Pro Pro Leu Val Gly Thr Pro Val Lys Ile Gly Glu Gly Met Leu
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Ala Asp Phe Val Ser Gln Ala Ser Pro Met Ile Pro Ala Ile Val Val
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Ser Cys Val Asn Glu Ile Glu Gln Arg Gly Leu Thr Glu Ala Gly Leu
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Tyr Arg Ile Ser Gly Cys Asp Arg Thr Val Lys Glu Leu Lys Glu Lys
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Phe Leu Lys Val Lys Thr Val Pro Leu Leu Ser Lys Val Asp Asp Ile
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His Val Ile Cys Ser Leu Leu Lys Asp Phe Leu Arg Asn Leu Lys Glu
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Pro Leu Leu Thr Phe Trp Leu Ser Lys Ala Phe Met Glu Ala Ala Glu
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Ile Thr Asp Glu Asp Asn Ser Thr Ala Ala Met Tyr Gln Ala Val Ser
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Glu Leu Pro Gln Ala Asn Arg Asp Thr Leu Ala Phe Leu Met Ile His
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Leu Gln Arg Val Ser Gln Ser Pro Asp Thr Lys Met Asp Ile Ala Asn
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13

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 Pro Asp Pro Val Thr Met Phe Gln Asp Ile Lys Arg Gln Leu Lys Val
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 Val Glu Arg Leu Leu Ser Leu Pro Leu Glu Tyr Trp Asn Gln Phe Met
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 Met Val Asp Gln Glu Asn Ile Asp Ser Gln Arg Gly Asn Gly Asn Ser
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 Thr Pro Arg Thr Pro Asp Val Lys Val Ser Leu Leu Gly Pro Val Thr
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 Thr Pro Glu Phe Gln Leu Val Lys Thr Pro Leu Ser Ser Ser Leu Ser
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Lys Asp Ile Gly Met Phe His Leu Ile Asp Glu Ile Glu Arg Leu Arg	
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aag ctg tgg aaa gat tcc gag gaa tcc aaa aag cgg ctg aat gca gat	192
Lys Leu Trp Lys Asp Ser Glu Glu Ser Lys Lys Arg Leu Asn Ala Asp	
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14

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Arg Glu Lys Gln Leu Lys Asp Ala Met Lys Asn Gly Ile Phe Asn Ser	
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Gly Thr Pro Pro Lys Arg Cys Arg Asp Asp Gly Ser Thr Pro His Gln	
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Thr Phe Ile Glu Ala Gly Ile Lys Ala Met Arg Lys Cys Asp Lys Cys	
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Phe Leu Arg Asp Leu Lys Asp Pro Leu Ile Pro Arg Thr Ser Arg Gln	
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17

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 485 490 495
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 515 520 525
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 Glu Phe Asp Asp Val Tyr Trp Gln Arg Phe Leu Gly Thr Ser Ala Val
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 Ser Met Ala Ser Asn Gln Ile Glu Thr Ala Arg His Gln Asp Asn Phe
 625 630 635 640

19

Ala Leu Cys Asp Arg Ser Ile Leu Gly Pro Val Thr Thr Ser Pro Ala
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Thr Pro Leu Leu Ala Arg Ser Ala Asn Ala Thr Arg Ala Arg Gly Ala
660 665 670

His Leu Leu Gly Ser Met Phe His Asp
675 680

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